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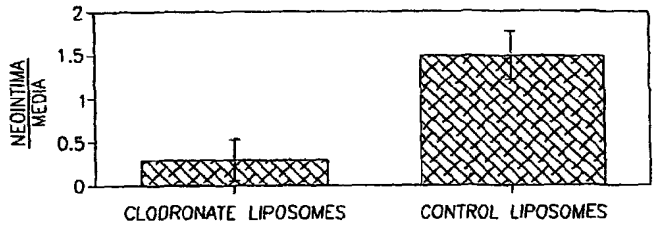
(54) Title: METHOD OF INHIBITING RESTENOSIS



(57) Abstract: A method of treating or preventing restenosis by administering to an individual an effective amount of an active compound which depletes and/or inhibits blood monocytes or macrophages thereby treating restenosis. A method of inhibiting the activity or production of cytokines or growth factors associated with vascular restenosis, by administering to an individual an effective amount of an active compound which depletes and/or inhibits blood monocytes or macrophages thereby treating restenosis. The cytokines and growth factors include, but are not limited to interleukin 1-β, matrix metalloproteinase-2, and platelet-derived growth factor β (PDGFβ). The active compound may be administered directly, encapsulated in a carrier particle, or in a particulate dosage form. The compound may be encapsulated, embedded or adsorbed within the particle, dispersed uniformly in the polymer matrix, adsorbed on the particle surface, or in combination of any of these forms. The particles include liposomes or inert polymeric particles, such as microcapsules, nanocapsules, nanoparticles, nanospheres, or microparticles. The particulates include any suspended or dispersed form of the compound which is not encapsulated, entrapped, or adsorbed within a polymeric particle. The particulates include suspended or dispersed colloids, aggregates, flocculates, insoluble salts and insoluble complexes of the active compound. The compound includes any intra-cellular toxin which depletes and/or inhibits blood monocytes or macrophages, such as, for example, a bisphosphonate.

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METHOD OF INHIBITING RESTENOSIS

FIELD OF THE INVENTION

The present invention is concerned with compositions capable of preventing, inhibiting or reducing restenosis (sometimes referred to in the art as *"accelerated arteriosclerosis"* and *"post-angioplasty narrowing"*). Specifically, the invention relates to the use of compounds which can selectively inhibit and/or deplete macrophages and monocytes, thereby preventing or reducing restenosis. One preferred class of compounds are bisphosphonates.

BACKGROUND OF THE INVENTION

Over the past decade, mechanical means of achieving revascularization of obstructive atherosclerotic vessels have been greatly improved. Percutaneous transluminal coronary angioplasty (PTCA) procedures include, but are not limited to, balloon dilatation, excisional atherectomy, endoluminal stenting, rotablation and laser ablation. However, revascularization induces thrombosis, and neointimal hyperplasia, which in turn cause restenosis in a substantial proportion of coronary arteries after successful balloon angioplasty and in aortacoronary saphenous vein bypass graft and other coronary grafts. Furthermore, intimal hyperplasia causes restenosis in many superficial femoral angioplasties, carotid endarterectomies, and femoro-distal vein bypasses. Restenosis is the formation of new blockages at the site of the angioplasty or stent placement or the anastomosis of the bypass. As a result, the patient is placed at risk of a variety of complications, including heart attack or other ischemic disease, pulmonary embolism and stroke. Thus, such procedures can entail the risk of precisely the problems that its use was intended to ameliorate. The introduction of endovascular stents has reduced the incidence of restenosis, but this problem still remains significant, since restenosis or "over exuberant" tissue healing may occur at the site of stent placement. (Waller, B.F. et al., 1997, Clin-Cardiol., 20(2):153-60; Anderson, W.D et al., 1996, Curr-Opin-Cardiol., 11(6):583-90; Moorman, D.L. et al., 1996, Aviat-Space- Environ-Med., 67(10):990-6; Laurent, S. et al., 1996, Fundam. Clin. Pharmacol., 10(3):243-57;

Walsh, K. et al., 1996, Semin-Interv-Cardiol., 1(3):173-9; Schwartz, R.S., 1997, Semin-Interv-Cardiol., 2(2):83-8; Allaire, E. et al., 1997, Ann. Thorac. Surg., 63:582-591; Hamon, M. et al., 1995, Eur. Heart J., 16:33s-48s; Gottsauner-Wolf, M., et al., 1996, Clin. Cardiol., 19:347-356).

Despite extensive research on the incidence, timing, mechanisms and pharmacological interventions in humans and animal models to date, no therapy exists which consistently prevents coronary restenosis (Herrman, J.P.R. et al., 1993, Drugs, 46:18-52; Leclerc, G. et al., 1995, Elsevier Science, 722-724, Topol, E., 1997, The NY Academy of Sciences, 225-277). Compositions and methods for the reduction or prevention of restenosis are still greatly desired. Accordingly, it would be desirable to develop novel compositions and methods that are effective in treating restenosis and preventing its reoccurrence.

SUMMARY OF THE INVENTION

The present invention relates to a method of treating or preventing restenosis by administering to an individual a compound which inhibits and/or depletes blood monocytes and tissue macrophages, thereby treating or preventing restenosis. The active compound is an intra-cellular toxin that directly enters or is released within the targeted macrophage/monocytes and inhibits and/or destroys the macrophage and monocytes.

In one embodiment, the present invention relates to a method of treating or preventing restenosis by directly administering to an individual an effective amount of compound which specifically targets monocytes and macrophage and is able to inhibit the activity of and/or destroy the monocytes and macrophages.

In a further embodiment, the present invention relates to a method of treating or preventing restenosis by administering to an individual an effective amount of a formulation comprising an encapsulated compound. The term "encapsulated compound" includes a compound which is encapsulated, embedded, and/or adsorbed within a particle, dispersed in the particle matrix, adsorbed or linked on the particle surface, or a combination of any of these forms. The particles include, but are not limited to, inert polymeric particles, such as microcapsules, nanocapsules, nanospheres, microspheres, nanoparticles, microparticles, or liposomes.

In a further embodiment, the present invention relates to a method of treating or preventing restenosis by administering to an individual an effective amount of a formulation comprising a particulate compound. The particulates include, but are not limited to aggregates, flocculates, colloids, polymer chains, insoluble salts and insoluble complexes of the compound.

The active compound of the present invention includes any intra-cellular toxin that once enters or is released within the macrophage/monocyte and inhibits the activity of and/or kills the macrophage or monocyte. One preferred class of compounds are bisphosphonates. Other effective compounds include, but are not limited to selenium, gallium, gadolinium, and gold.

Effective phagocytosis of both the encapsulated compound and the particulate compound by the monocytes/macrophages can affect the activity of such phagocytic cells. The compound affects restenosis by inhibiting phagocytic cells involved in the restenotic cascade, such as macrophages/monocytes and fibroblasts. The delivery system affects smooth-muscle cells (SMC) and extracellular matrix production indirectly by inhibiting the cells that trigger their migration and/or proliferation. Nevertheless, a direct effect on SMC may also occur. The formulation may be administered by any route which effectively transports the active compound to the desirable site of action. In a preferred embodiment, the mode of administration includes intra-arterial, intravenous or subcutaneous administration.

In a further embodiment, the present invention includes a pharmaceutical composition comprising a formulation selected from the group consisting of a compound, an encapsulated compound, and a particulate compound, together with a pharmaceutically acceptable carrier, stabilizer or diluent for the prevention or treatment of vascular restenosis.

In yet a further embodiment, the present invention includes a method of inhibiting the activity and/or production of cytokines and growth factors associated with vascular restenosis, by administering to an individual an effective amount of a formulation comprising an encapsulated compound, thereby inhibiting restenosis.

In still yet a further embodiment, the present invention includes a method of inhibiting the activity and/or production of cytokines and growth factors associated

with vascular restenosis, by administering to an individual an effective amount of a formulation comprising a particulate compound, thereby inhibiting restenosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1-3 are bar graphs of results demonstrating the effect of clodronate encapsulated in liposomes on the reduction of restenosis in an experimental rat carotid catheter injury model as compared to the effect of control liposomes which did not contain clodronate on the same rats. In these figures:

Figure 1 shows the mean neointimal area to the area of the media in rats treated with clodronate containing liposomes as compared to rats treated with control liposomes. The medial area is the difference between the total arterial area and the original lumen area.

Figure 2 shows the % stenosis in rats treated with clodronate containing liposomes as compared to the % stenosis in rats treated with control liposomes.

Figure 3 shows the extent of medial area as an indirect index of smooth muscle cell viability and determined as the difference between the total arterial area and the original lumen area (External elastic lamina bound area – Internal elastic lamina bound area) in rats treated with clodronate containing liposomes as compared to rats treated with control liposomes only.

Figure 4 illustrates the antirestenotic effects of liposomal clodronate in the balloon-injured rat and atherosclerotic rabbit carotid arterial models.

Figure 5 tabulates the characteristics of a typical formulation of ISA encapsulated nanoparticles.

Figure 6 illustrates the effect of ISA, specifically, free ISA, Ca^{+2}-ISA salt, and ISA encapsulated in nanoparticles, on the growth of RAW 264 cells in vitro.

Figure 7 illustrates the effect of 50 μM Ca^{+2}-ISA salt formulations, specifically, Ca^{+2}-ISA adsorbed on blank NP, Ca^{+2}-ISA salt and polyvinyl alcohol (PVA), and Ca^{+2}-ISA salt, on the proliferation of RAW 264 cells in vitro.

Figures 8a and 8b are bar graphs of results demonstrating the effect of ISA encapsulated in nanoparticles on the reduction of restenosis in an experimental rat

carotid catheter injury model as compared to the effect of control nanoparticles (i.e., blank NP) which did not contain ISA, on the same rats. In these figures:

Figure 8a illustrates the % stenosis in rats treated with ISA encapsulated nanoparticles as compared to the % stenosis in rats treated with control nanoparticles; and

Figure 8b illustrates the mean neointimal area to the area of the media ratio in rats treated with ISA encapsulated in nanoparticles as compared to rats treated with control nanoparticles. The medial area is the difference between the total arterial area and the original lumen area.

Figure 9 tabulates the characteristics of a typical formulation of alendronate encapsulated in nanoparticles.

Figure 10 tabulates the effect of alendronate loaded nanoparticles on the proliferation of RAW 264 cells.

Figures 11, 12a and 12b are bar graphs of results demonstrating the effect of alendronate encapsulated in nanoparticles on the reduction of restenosis in a hypercholesterolemic balloon-injured rabbit model as compared to the effect of control nanoparticles which did not contain alendronate on the same rats via subcutaneous administration. The graphs also compare the effect of subcutaneous (SC) and intravenous (IV) administration in reducing restenosis. In these figures:

Figure 11 illustrates the % stenosis in rats treated with alendronate loaded nanoparticles as compared to the % stenosis in rats treated with control nanoparticles, wherein the particles were administered subcutaneously;

Figure 12a compares the % stenosis in rats treated with: 1.5 mg/kg of alendronate loaded nanoparticles via SC administration, 0.15 mg/kg of alendronate loaded nanoparticles via SC administration and 0.15 mg/kg of alendronate loaded nanoparticles via IV administration; and

Figure 12b illustrates the mean neointimal to medial area ratio (N/M) in rats treated with ISA loaded nanoparticles as compared to rats treated with control nanoparticles, and also compares the mean neointimal area to medial area ratio (N/M) in rats treated with: 1.5 mg/kg of alendronate loaded nanoparticles via SC administration, 0.15 mg/kg of alendronate loaded nanoparticles via SC

administration and 0.15 mg/kg of alendronate loaded nanoparticles via IV administration. The medial area is the difference between the total arterial area and the original lumen area.

Figure 13 illustrates the effect of alendronate encapsulated in nanoparticles on the number of monocytes in the human blood following incubation for 24 hours.

Figures 14a, 14b, 14c and 14d illustrate the effect of liposomal clodronate treatment on interleukin 1-β (IL-1β) concentration and matrix metalloproteinase-2 (MMP-2) activity in the arteries of rats and rabbits following balloon injury. Specifically, Figures 14a and 14b illustrate the effect of liposomal clodronate treatment on IL-1β concentration in the rat and rabbit models, respectively, and Figures 14c and 14d illustrate the effect of liposomal clodronate treatment on MMP-2 activity in the rat and rabbit models, respectively.

Figure 15 illustrates the effect of liposomal clodronate on IL-1β transcription in rabbits' arteries following balloon injury.

Figures 16a, 16b, and 16c illustrate the effect of liposomal clodronate treatment on the platelet-derived growth factor (PDGF) system in the arterial walls of rats following balloon injury. Specifically, Figure 16a illustrates the effect on platelet-derived growth factor β receptor (PDGFβR) activation (i.e., tyrosine phosphorylation), Figure 16b illustrates the effect on the PDGFβR protein, and Figure 16c illustrates the effect on the PDGF-B protein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for reducing, delaying or eliminating restenosis. Reducing restenosis includes decreasing the thickening of the inner blood vessel lining that results from stimulation and proliferation of smooth muscle cell and other cell migration and proliferation, and from extracellular matrix accumulation, following various angioplasty procedures. Delaying restenosis includes delaying the time until angiographic re-narrowing of the vessel appears or until the onset of clinical symptoms which are attributed to stenosis of this vessel. Eliminating restenosis following angioplasty includes reducing hyperplasia to an extent which is less than 50% of the vascular lumen, with

lack of clinical symptoms of restenosis. Methods of intervening include re-establishing a suitable blood flow through the vessel by methods such as, for example, repeat angioplasty and/or stent placement, or coronary artery bypass graft (CABG).

The present invention includes a method of treating or preventing restenosis by administering to an individual, an effective amount of any compound or formulation known to inactivate or inhibit blood monocytes and tissue macrophages. Any compound or formulation that selectively targets macrophage and monocytes and inhibits and/or depletes them would be effective in treating or preventing restenosis. The compound may be administered directly, encapsulated in a particle, or in particulate form. The compound may be administered directly if it can selectively target monocytes and macrophages and inhibit their activity. If, however, the compound is unable to selectively target the monocytes and macrophages, i.e., if it is unable to cross the macrophage/monocyte cellular membrane or if it may target many different types of cells, it may be encapsulated in a particle carrier or formulated in particulate form having a specifically sized dimension. The compounds when either encapsulated in liposomes, microparticles or nanoparticles of a specific size or when in a particulate form, such as, for example, in aggregates of a specific size, specifically target and are efficiently engulfed by way of phagocytosis by the macrophage and monocytes. In contrast, non-phagocytic cells are incapable of taking up the encapsulated compound and particulate compound due to their specifically sized dimension.

In one embodiment, the formulation comprises the active compound encapsulated in a particle of different material, hereinafter referred to as an "encapsulated compound." The term "encapsulated compound" includes an active compound which is encapsulated, embedded, and/or adsorbed within a particle, dispersed in the particle matrix, adsorbed or linked on the particle surface, or in combination of any of these forms. The particle includes any of the liposomes, microparticles, nanoparticles, nanospheres, microspheres, microcapsules, or nanocapsules known in the art (M. Donbrow in: Microencapsulation and Nanoparticles in Medicine and Pharmacy, CRC Press, Boca Raton, FL, 347, 1991).

The term particle includes both polymeric and non-polymeric preparations of the active compound. In addition, suspending agents and stabilizers may also be used with the encapsulated compound formulation.

In a further embodiment, the formulation comprises an active compound in particulate form, hereinafter referred to as a "particulate compound." The "particulate compound" formulation includes, for example, an insoluble salt, insoluble ester, or insoluble complex of the active compound. Typically, "insoluble" refers to a solubility of one (1) part of a compound in more than ten-thousand (10,000) parts of a solvent. A "particulate compound" dosage form includes any insoluble suspended or dispersed particulate form of the active compound which is not encapsulated, entrapped or adsorbed within a polymeric particle. Particulate compounds include, but are not limited to, suspended or dispersed colloids, aggregates, flocculates, insoluble salts, insoluble complexes, and polymeric chains of the active compound. In addition, suspending agents and stabilizers may be used with the particulate compound formulation.

The formulation of the present invention, for example, the encapsulated compound or the particulate compound, inhibits smooth muscle cell migration and proliferation by transiently depleting and/or inactivating cells that are important triggers in the restenosis cascade, namely macrophages and/or monocytes. The active compound, when encapsulated in liposomes or nanoparticles, or when in a particulate dosage form, such as, for example, in aggregates of a specific size, are taken-up, by way of phagocytosis, very efficiently by the macrophages and monocytes, and to some extent by other cells with phagocytic activity such as fibroblasts. Once inside the macrophages, the structure of the carrier particle (e.g., liposome, microparticle, nanoparticle) is disrupted and the active compound is released, thereby inhibiting the activity and/or killing the macrophages. Since macrophages, in their normal state, are recruited to the areas traumatized by angioplasty or other intrusive intervention and initiate the proliferation of smooth-muscle cells (SMC), inhibiting the macrophages' activity inhibits the migration and proliferation of SMC. After being taken-up by the macrophages, the active compound has a sustained inhibitory activity on the macrophages. Thus, prolonged

release of the active compound is not required in order to sustain inhibition. Accordingly, the method of inhibiting or reducing restenosis by administering an active compound as an encapsualted compound or as a particulate compound is preferably a systemic therapy, in that the encapsulated compound and particulate compound target the circulating monocytes and macrophages.

It should be noted, however, that some encapsulated compounds and particulate compounds may have a direct effect on SMC activity. Additionally, some of the encapsulated compounds and particulate compounds may also inactivate other phagocytic cells and cells of the white-blood cell lineage in the body, such as liver and spleen macrophages and macrophages in the arterial walls.

Furthermore, the delivery system of the present invention not only retains the active compound for a sufficient time so that the active compound is not released in the body fluids, but also efficiently discharges the drug within the target cell. The active compound is formulated into "encapsulated compounds" or "particulate compounds" because, in most instances, the free active compound is ineffective since it is not taken-up by phagocytic cells. Encapsulating the active compound in carrier particles of a specific size or formulating the active compound into particulates of a specific size allows the formulations to be taken-up effectively and efficiently by the macrophage and monocytes. The formulation is preferably in the size range of 0.1-1.0 microns. However, this is merely an example and other size ranges may be used without departing from the spirit or scope of the invention.

The present invention also provides a method of inhibiting the activity, production, and/or transcription of certain cytokines and growth factors that are associated with restenosis or with any of the cell types involved in the restenotic cascade, by administering a compound, an encapsulated compound, or a particulate compound. The select cytokines and growth factors that are associated with restenosis or with the cell types involved in the restenotic cascade include, but are not limited to, interleukin-1 (IL-1), matrix metalloproteinases (MMPs), and platelet-derived growth factor β (PDGFβ). For example, IL-1β and MMP-2 are major products of activated macrophages, that are secreted following arterial injury, and contribute to the process of neointimal proliferation. Additionally, PDGF-BB is a

strong chemoattractant for vascular smooth muscle cells and is involved in neointima formation secondary to vascular injury. (Fishbein, I., et al., 2000, Arterioscler. Thromb. Vasc. Biol., 20:667-676; Jawien, A., et al., 1992, J. Clin. Invest., 507-511; Ross, R., 1993, Nature, 362:801-809; Panek, R.L., et al., 1997, Arterioscler. Thromb. Vasc. Biol., 17:1283-1288; Waltenberger, J, 1997, Circulation, 96:4083-4094; Deguchi, J., et al., 1999, Gene Ther., 6:956-965.)

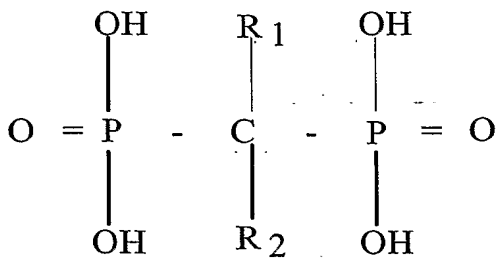
The active compound includes any substance that directly enters or is released within the targeted macrophage/monocytes and inhibits and/or destroys the macrophage and monocytes. In accordance with the present invention, the active compound may comprise an intra-cellular toxin. The term "active compound" encompasses in its scope, not only monomer, but also polymeric chains of the compound. One preferred class of compounds are bisphosphonates. Free bisphosphonates, due to their affinity to bone and due to their inability to cross cellular membranes, have virtually no effect on the inflammatory response. In contrast, bisphosphonates, when encapsulate in liposomes, microparticles or nanoparticles of specific dimensions in an encapsulated bisphosphonate form, or when in a particulate bisphosphonate dosage form, such as, for example, in aggregates of a specific size, are specifically targeted to, and efficiently taken-up by way of phagocytosis, by the macrophages and monocytes. In contrast, non-phagocytic cells are incapable of taking-up the encapsulated bisphosphonate and particulate bisphosphonate

While the foregoing and following detailed description often relates to a preferred embodiment of the present invention, i.e., the use of the active compound, a bisphosphonate, specifically, encapsulated bisphosphonate or bisphosphonate particles, it will be understood by the skilled practitioner in the art that any compound or intra-cellular toxin that can effectively deplete and/or inactivate monocytes/macrophages can be employed without departing from the spirit or scope of the invention. For instance, gallium and gold are inactivators of monocytes and macrophages and can be used to treat restenosis. Other effective compounds include, but are not limited to, selenium, gadolinium, silica, mithramycin, paclitaxol,

sirolimus, and everolimus. Furthermore, the intra-cellular toxin can be formulated as an encapsualted intra-cellular toxin or a particulate intra-cellular toxin.

In accordance with the present invention, a preferred class of active compounds are bisphosphonates. Bisphosphonates ("BPs") (formerly called diphosphonates) are compounds characterized by two C-P bonds. If the two bonds are located on the same carbon atom (P-C-P) they are termed geminal bisphosphonates. The BPs are analogs of the endogenous inorganic pyrophosphate which is involved in the regulation of bone formation and resorption. The term bisphosphonates is generally used for geminal and non-geminal bisphosphonates. The BPs may at times form polymeric chains. BPs act on bone because of their affinity for bone mineral and also because they are potent inhibitors of bone resorption and ectopic calcification. BPs have been clinically used mainly as (a) antiosteolytic agents in patients with increased bone destruction, especially Paget's disease, tumor bone disease and osteoporosis; (b) skeletal markers for diagnostic purposes (linked to ^{99m}Tc); (c) inhibitors of calcification in patients with ectopic calcification and ossification, and (d) antitartar agents added to toothpaste (Fleisch, H., 1997, in: Bisphosphonates in bone disease. Parthenon Publishing Group Inc., 184-186). Furthermore, being highly hydrophilic and negatively charged, BPs in their free form are almost incapable of crossing cellular membranes.

The bisphosphonates, when encapsualted in polymeric particles of specific dimensions, or when in a particulate dosage form, such as, for example, in aggregates of a specific size, are used for treatment or prevention of vascular restenosis. The term bisphosphonate as used herein, denotes both geminal and non-geminal bisphosphonates. A preferred active compound has the following formula (I):



(I)

wherein R_1 is H, OH or a halogen atom; and

R_2 is halogen; linear or branched C_1-C_{10} alkyl or C_2-C_{10} alkenyl optionally substituted by heteroaryl or heterocyclyl C_1-C_{10} alkylamino or C_3-C_8 cycloalkylamino where the amino may be a primary, secondary or tertiary; -NHY where Y is hydrogen, C_3-C_8 cycloalkyl, aryl or heteroaryl; or R_2 is -SZ where Z is chlorosubstituted phenyl or pyridinyl.

The present invention thus provides the use of said active compound, a complex of the active compound, a formulation of the active compound or a pharmaceutically acceptable salt or ester thereof for the preparation of a composition for the prevention or treatment of vascular restenosis.

The present invention still further provides a pharmaceutical composition for the prevention or treatment of restenosis comprising, an effective amount of the active compound, a formulation of the active compound, specifically, an encapsulated compound or particulate compound, optionally together with a pharmaceutically acceptable carrier or diluent.

The term "effective amount" denotes an amount of the compound or formulation, which is effective in achieving the desired therapeutic result, namely prevention, reduction, or elimination of vascular restenosis. The effective amount may depend on a number of factors including: weight and gender of the treated individual; the type of medical procedure, e.g. whether the vascular restenosis to be inhibited is following balloon angioplasty, balloon angioplasty followed by deployment of a stent; the mode of administration of the formulation (namely whether it is administered systemically or directly to the site); the type of carrier being used (e.g. whether it is a carrier that rapidly releases the active compound or a carrier that releases it over a period of time); the therapeutic regime (e.g. whether the formulation is administered once daily, several times a day, once every few days, or in a single dose); clinical indicators of inflammation; clinical factors influencing the rate of development of restenosis such as diabetes, smoking, hypercholesterolemia, renal diseases; anatomical factors such as whether there is severe preangioplasty stenosis, total occlusion, left anterior descending coronary artery location, saphenous vein graft lesion, long lesions, multivessel or multilesion PTCA; and on the dosage

form of the composition. Moreover, procedural variables may also have bearing on the dosage, such as greater residual stenosis following PTCA, severe dissection, intimal tear, appropriate size of balloon, and the presence of thrombus. The artisan, by routine type experimentation should have no substantial difficulties in determining the effective amount in each case.

The invention is applicable for the prevention, reduction or treatment of vascular restenosis and mainly, but not limited to, coronary restenosis after angioplasty. Vascular restenosis primarily results from various angioplasty procedures including balloon angioplasty, intravascular stent deployment or other methods of percutaneous angioplasty (including angioplasty of coronary arteries, carotid arteries, and other vessels amenable for angioplasty) as well as for restenosis resulting from vascular graft stenosis (e.g. following by-pass surgery) (Braunwald, E., 1997, Heart Disease in: A textbook of cardiovascular medicine; 5th Ed., W.B. Saunders Company: Philadelphia).

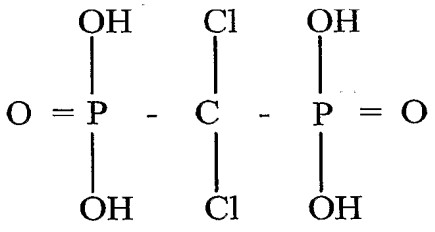
In addition, the invention is also applicable for use in prevention, reduction or treatment of vascular restenosis in peripheral arteries and veins.

One exemplary application of the invention is to prevent and treat in-stent restenosis. It is a widely acceptable medical procedure to deploy a stent within a blood vessel within the framework of an angioplastic procedure, to support the walls of the blood vessel. However, very often restenosis occurs notwithstanding the presence of the stent within the blood vessel. In accordance with the invention, the above noted active compound or formulation may be administered, either systemically or directly to the site, in order to prevent or inhibit such restenosis. The active compound may be formulated in a manner allowing its incorporation onto the stent which, in fact, yields administration of said active compound directly at the site. The formulation may be devised in that manner, for example, by including it within a coating of the stent. Examples of coatings are polymer coatings, (e.g., made of polyurethane), gels, fibrin gels, hydrogels, carbohydrates, gelatin, or any other biocompatible gel.

The formulation used in accordance with the invention may be prepared into pharmaceutical compositions by any of the conventional techniques known in the art

(see for example, Alfonso, G. et al., 1995, in: The Science and Practice of Pharmacy, Mack Publishing, Easton PA, 19th ed.). The compositions may be prepared in various forms suitable for injection, instillation or implantation in the body such as suspensions of the nanoparticles, as in a coating of a medical device such as a stent (see above). In addition, the pharmaceutical compositions of the invention may be formulated with appropriate pharmaceutical additives for parental dosage forms. The preferred administration form in each case depends on the desired delivery mode, which is usually that which is the most physiologically compatible with the patient's condition and with the other therapeutic treatments which the patient currently receives.

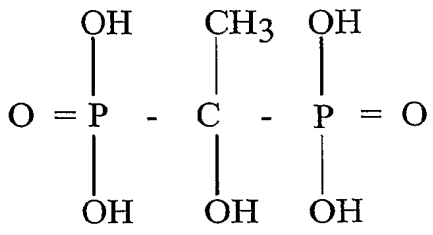
As presented *supra*, in a preferred embodiment of the invention, the active compound is selected from the group of bisphosphonates. One active compound for this group is the compound clodronate, (dichloromethylene) diphosphonic acid, (Fleisch, H., 1997, in: Bisphosphonates in bone disease. Parthenon Publishing Group Inc., 184-186) having the following formula (II):



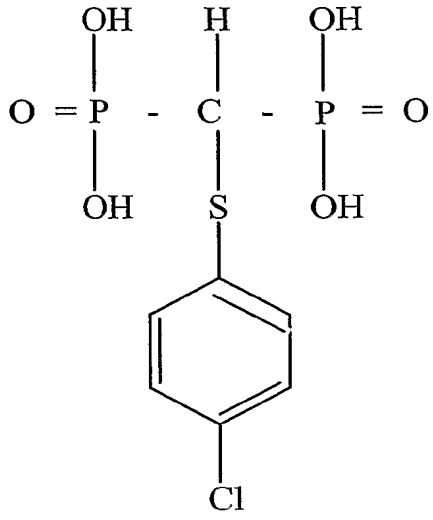
(II)

Clodronate was previously described for use in the treatment of hypercalcemia resulting from malignancy in the treatment of tumor associated osteolysis (Fleisch, H., 1997, in: Bisphosphonates in bone disease. Parthenon Publishing Group Inc., 184-186). Clodronate was also found to inhibit macrophages *in vitro* and to suppress macrophage activity in the spleen and liver tissues of mice. (Mönkkönen, J. et al, 1994, J. Drug Target, 2:299-308; Mönkkönen, J.et al., 1993, Calcif. Tissue Int., 53:139-145).

Other preferred active compounds of this group are etidronate and tiludronate having the following formulae (III) and (IV) respectively:



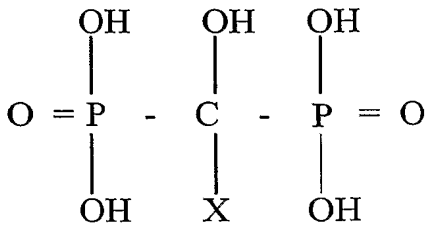
(III)



(IV)

Additional BPs having activities similar to that of clodronate are also preferred in accordance with the invention. Such BPs may be selected on the basis of their ability to mimic the biological activity of clodronate. This includes, for example: *in vitro* activity in inhibiting phagocytic activity of phagocytic cells, e.g. macrophages and fibroblasts; inhibition of secretion of IL-1 and/or IL-6 and/or TNF-α from macrophages; reduction of MMP activity, for example, MMP-2 activity; inhibition of PDGFβR activation and/or reduction of PDGF-B protein levels; and, *in vivo* activity, e.g. the ability of the tested formulation to prevent or reduce restenosis in an experimental animal model such as, for example, the rat or rabbit carotid catheter injury model described in Example 1 below, or porcine model of restenosis.

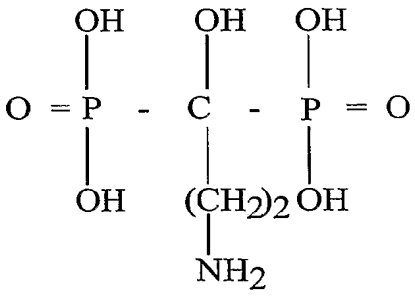
The most preferred group of active compounds in accordance with the invention are the amino-BPs and any other nitrogen-containing BPs having the following general formula (V):



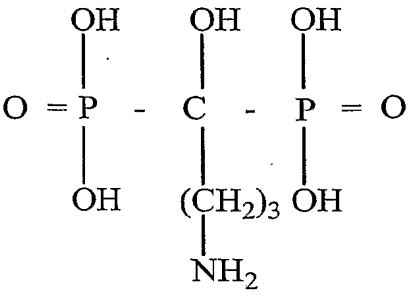
(V)

wherein X represents C_1-C_{10} alkylamino or C_3-C_8 cycloalkylamino, where the amino may be primary, secondary or tertiary; or X represents NHY where Y is hydrogen, C_3-C_8 cycloalkyl, aryl or heteroaryl.

The BPs belonging to this group are believed not to be metabolized and have been shown at relatively low concentrations to induce secretion of the interleukin, IL-1, and cause, at relatively high concentrations, apoptosis in macrophages (Mönkkönen, J.et al., 1993, Calcif. Tissue Int., 53:139-145). Preferred BPs belonging to this group are for example, pamidronate and alendronate having the following formulae (VI) and (VII), respectively.



(VI)



(VII)

Although the geminal BPs are preferred BPs in accordance with the invention, non-geminal BPs, monophosphonates of BPs, termed generally as phosphonates may also be used as active compounds in accordance with the invention.

Additional bisphosphonates include, but are not limited to, 3-(N,N-dimethylamino)-1-hydroxypropane-1,1-diphosphonic acid, e.g. dimethyl-APD; 1-hydroxy-ethylidene-1,1-bisphosphonic acid, e.g. etidronate; 1-hydroxy-3-(methylpentylamino)-propylidene-bisphosphonic acid, (ibandronic acid), e.g. ibandronate; 6-amino- 1-hydroxyhexane-1,1-diphosphonic acid, e.g. amino-hexyl-BP; 3-(N-methyl-N-pentylamino)-1-hydroxypropane-1,l-diphosphonic acid, e.g. methyl-pentyl-APD; 1-hydroxy-2-(imidazol-1-yl)ethane-1,1-diphosphonic acid, e.g. zoledronic acid; 1-hydroxy-2-(3-pyridyl)ethane-l,l-diphosphonic acid (risedronic acid), e.g. risedronate; 3-[N-(2-phenylthioethyl)-N-methylamino]-l-hydroxypropane-1,1-bishosphonic acid; 1-hydroxy-3-(pyrrolidin-1-yl)propane-1,1-bisphosphonic acid, 1-(N-phenylaminothiocarbonyl)methane-l,l-diphosphonic acid, e.g. FR 78844 (Fujisawa); 5-benzoyl-3,4-dihydro-2H-pyrazole-3,3-diphosphonic acid tetraethyl ester, e.g. U81581 (Upjohn); and 1-hydroxy-2-(imidazo[1,2-a]pyridin-3-yl)ethane-1,1-diphosphonic acid, e.g. YM 529.

Thus, suitable bisphosphonates for use in the present invention include the acid compounds presented above, any acceptable salts thereof, and crystalline and amorphous BPs. Additionally, preferred bisphosphonates are the amino-bisphosphonates such as alendronate, zolendronate, and risendronate.

The formulation of the invention may comprise said active compound either encapsulated within a carrier particle, adsorbed on the particle surface, complexed with metal cations such as calcium, magnesium or organic bases, formed into insoluble salts or complexes, or polymerized to yield polymers of up to 40 monomers. The salts may be sodium, potassium, ammonium, gallium or calcium salts or salts formed with any other suitable cation (e.g. organic amino compounds). The salts or polymers may be in a micronized particulate form having a diameter within the range of about 0.01-1.0 μm, preferably within a range of about 0.1-0.5 μm. The active compounds in their salt form may be with or without water of

crystallization (hydrous and anhydrous). Additionally, additives such as polyvinyl alcohol (PVA), pluronics, and other surface active agents, may be used to stabilize the salt and or complex to establish a colloidal or nano-size suspension. In one embodiment for example, the composition may comprise a Ca-BP salt and or complex.

In one embodiment of the invention, the active compound is encapsulated in liposomes. The liposomes may be prepared by any of the methods known in the art (regarding liposome preparation methods see Mönkkönen, J. et al, 1994, J. Drug Target, 2:299-308, and Mönkkönen, J.et al., 1993, Calcif. Tissue Int., 53:139-145). The liposomes may be positively charged, neutral or negatively charged (negatively charged liposomes being currently preferred), and may be single or multilamellar. Suitable liposomes in accordance with the invention are preferably non toxic liposomes such as, for example, those prepared from phosphatidyl-choline phosphoglycerol, and cholesterol, e.g. as described below. In many cases, use of liposomal delivery results in enhanced uptake of the active compound by cells via phagocytosis. The diameter of the preferred liposomes may range from 0.15 to 300 nm. However, this is merely a non-limiting example, and liposomes of other size ranges may also be used.

In a further preferred embodiment, the active compound, for example, the bisphosphonate, may be encapsulated or embedded in inert particles. In yet a further embodiment, the active compound may be adsorbed onto the surface of, or adsorbed within, a blank particle, wherein a blank particle is a particle which has no drug encapsulated or embedded therein. Alternatively, the active compound may form a particulate, which includes a colloid, aggregate, flocculate or other such structure known in the art for the preparation of particulates of drugs. Furthermore, such particulates may be aggregates of the polymerized active compound.

Particulates of the active compound may be obtained by using an insoluble salt or complex that can be obtained *in-situ,* i.e., starting with the soluble drug and "salting-out" the drug by adding for example, Ca at the appropriate concentration and pH. The dispersed or free particulates are formed and then stabilized by the aid of surface active agents, suspending agents, deflocculating agents or by thickening

agents, such as those used in gels. The active compound may be further precipitated by adding a trivalent cation, for example, gallium, thereby forming a precipitate of gallium-BP salt/complex.

The active compound may be encapsulated within or adsorbed onto particles, e.g., nanoparticles by utilizing, for example, a modified nano-precipitation method. In this embodiment of the invention, the polymeric nanoparticle containing the active compound is formed by mixing water and organic solutions of the drug and polymer (PLGA or other polymers), respectively. Thus, the nanoparticle containing drug formed is suspended in water and can be lyophilized. Additionally, the active compound may be entrapped or adsorbed into blank polymeric nanoparticles, and/or adsorbed on the surface of the blank polymeric nanoparticles. (Blank nanoparticles are particles which have no drug encapsulated, embedded, and/or adsorbed therein).

One advantage of particulate dosage forms of the active compound itself, or of polymeric particle dosage forms (e.g. nanoparticles), is the possibility of lyophilization and of sterilization methods other than filter-sterilization. Thus, these forms of the active compound have an extended shelf-life and ease of handling.

In a further embodiment, the active compound, for example, the bisphosphonate, may be encapsulated in nanoparticles ("NP"). Nanoparticles are 30-1000 nm diameter, spherical or non-spherical polymeric particles. The drug can be encapsulated in the nanoparticle, dispersed uniformly or non-uniformly in the polymer matrix (monolithic), adsorbed on the surface, or in combination of any of these forms. It is the submicron nature of this compositional form, which makes it more efficient in therapeutic applications. The submicron size facilitates uptake by phagocytic cells such as monocytes and macrophages, and avoids uptake in the lungs. In a preferred embodiment, the polymer used for fabricating nanoparticles is the biocompatible and biodegradable, poly(DL-lactide-co-glycolide) polymer (PLGA). However, any polymer which is biocompatible and biodegradable may be used. Therefore, additional polymers which may be used to fabricate the NP include, but are not limited to, polyanhydrides, polyalkyl-cyanoacrylates (such as polyisobutylcyanoacrylate), polyetheyleneglycols, polyethyleneoxides and their derivatives, chitosan, albumin, gelatin and the like. The size of the nanoparticle

used to encapsulate the active compound or bisphosphonate depends on the method of preparation and the mode of administration (e.g. IV, IA, etc.) Preferably, the nanoparticles range in size from 70-500 nm. However, depending on preparation and sterilization techniques, the more preferred ranges include, but are not limited to, 100-300 nm and 100-220 nm.

Encapsulating a small, hydrophilic, and charged active compound, for example, the bisphosphonate, in a nanoparticle is described herein. During the preparation of the nanoparticle, there is a rapid diffusion of the drug into the water phase, thus resulting in a low entrapment efficiency. Accordingly, a formulation was developed to overcome this low encapsulation efficiency. The following formulation parameters may influence drug entrapment efficiency and release properties: buffers, emulsifiers, stabilizers such as PVA, amount and molecular weight of PLGA polymers, type of BP, non-solvent type, timing, rate of mixing and evaporation of the ingredients, vacuum, and temperature. A cation, such as Ca^{+2}, a non-solvent, or other compounds may be incorporated with the BP, prior to encapsulation with the nanoparticle, in order to reduce the solubility of the hydrophilic drug and increase its entrapment efficiency. A "non-solvent" is immiscible with the polymer or drug but is miscible with the other solvent present and, as such, forces the polymer or drug to leave its solvent. Several methods of nanoparticle preparation known in the art may be used; the common methods including, but not limited to, emulsion or double-emulsion solvent-evaporation precipitation methods, nanoprecipitation methods, coacervation methods, and solid-lipid liposomal methods.

Since the nanoparticles' surfaces are preferably negatively charged due to the acidic functional groups of the polymer and/or the BP, increased uptake by phagocytic cells is expected, thereby leading to increased activity against restenosis. Although particles which are neutral in charge may also be used to encapsulate the BPs, the most efficient uptake by the monocytes/macrophages occurs with charged particles, with negatively charged particles being preferred.

The pharmaceutical carrier or diluent used in the composition of the invention may be any one of the conventional solid, liquid, or semisolid carriers known in the art. A solid carrier, for example, may be lactose, sucrose, gelatins, and

other carbohydrates. A liquid carrier, for example, may be a biocompatible oil suitable for injection such as peanut oil, water or mixtures of biocompatible liquids, or a biocompatible viscous carrier such as a polyethylene or gelatin gel.

The formulation of the active compound used for injection may be selected from emulsions, suspensions, colloidal solutions containing suitable additives, and additional suitable compositions known to the skilled artisan.

The formulation of the active compound may be administered by any route which effectively transports the active compound to the appropriate or desirable site of action. By a preferred embodiment of the invention, the modes of administration are intravenous (IV) and intra-arterial (IA) (particularly suitable for on-line administration). Other suitable modes of administration include intramuscular (IM), subcutaneous (SC), or intraperitonal (IP). Such administration may be bolus injections or infusions. The compositions may also be administered locally to the diseased site of the artery, for example, by means of a medical device which is coated with the active compound. Another mode of administration may be by perivascular delivery. Combinations of any of the above routes of administration may also be used in accordance with the invention.

The dosage of the active compound to be used also depends on the specific activity of the active compound selected, on the mode of administration (e.g. systemic administration or local delivery), the form of the active compound (e.g. polymer, encapsulated in a particle such as a liposome, nanoparticle etc.), the size of the particle, the type of bisphosphonate, the administration route, the number of injections, the timing of injections, the biology/pathology of the patient in need, and other factors as known *per se*.

In one embodiment, the dosage for clodronate-containing liposomes (liposomal clodronate, ("LC")) in humans preferably ranges from 0.015 mg/kg (per kg of body weight) to 150 mg/kg; more preferably, however, the dosage ranges from 0.15 to 15 mg/kg. Dosages outside these preferred ranges may also be used, as can be readily determined by the skilled artisan. When IV/IA injections or local delivery methods are used, i.e. via a balloon catheter, the dosage is at the lower end of the

range. However, when IM or SC administration modes are used the dosage is approximately 10 times that used for IV administration.

In accordance with a preferred embodiment of the invention, treatment of an individual with the formulation may be for the purpose of preventing restenosis before its occurrence. For prevention, the active compound may be administered to the individual before angioplasty procedure, during the procedure or after the procedure as well as combination of before, during and after procedural administration. Furthermore, the active compound may be administered via IV, IA, IM, SC, IP or any other suitable type of administration. For example, the active compound may be administered via IA the day of the angioplasty procedure (day 0), via IV the day before the procedure (-1) and/or on day 0, or both via IV the day before the procedure (-1) and also after the procedural administration, for example, on day 6.

In accordance with a further embodiment of the invention, the active compound is administered to an individual suffering from restenosis for the purpose of reducing or treating restenosis. In such a case, the active compound may also be administered to the individual at different periods of time after restenosis is discovered, either alone or in combination with other kinds of treatments.

In addition, the active compound may be administered before any other conditions which may yield accelerated arteriosclerosis, as well as acutely after the process has begun to inhibit further development of the condition.

EXAMPLES

The invention will now be demonstrated by way of non-limiting examples with reference to the accompanying drawings. The animal models used in the examples below include the balloon-injured rat carotid arterial model and the balloon-injured hypercholesterolemic rabbit carotid arterial model. The rat is an acceptable model in evaluating the antirestenotic effects of drugs and composites; however, the rabbit is more preferred since it, unlike the rat, is both atherosclerotic and contains a significant number of macrophages in the arterial wall.

EXAMPLE 1

Liposomes of clodronate

Stock solutions of clodronate were prepared by dissolving the drug in deionized water at a concentration of 0.11 M, pH = 7.

Liposome preparation

38.9 mg of distearoylphosphatidylglycerol (DSPG), 118.5 mg of distearoyl-phosphatidylcholine (DSPC) and 38.7 mg of cholesterol were accurately weighed and dissolved in 20 ml of chloroform: methanol (9:1) in a round bottom vial. The vial was gently warmed, and the solvent was then evaporated in rotavapor. 20 mls of hydrated diisopropylether were then added and the vial was put into a water bath until the contents were dissolved. 8 mls of the clodronate solution prepared as described above were then added, and the solution was sonicated at 55°C for a period of 45 minutes. The organic phase was then evaporated in rotavapor (55°C, 100 rpm). Similarly, other drug-containing liposomes can be prepared.

Purification of prepared liposomes

A Sephadex gel was prepared by dissolving 2.6 grams of Sephadex G-50 in 40 mls of water and stabilizing overnight. The column was rinsed with 100 mls of buffer (50 mM Mes + 50 mM HEPES + 75 mM NaCl, pH 7.2). The liposomes were applied to the column and the column was rinsed with the buffer. The liposome was seen as a band which can be followed in the column by its color. About 20 drops were collected from the column into each tube.

Animals

Animals were obtained and housed in the animal facilities of the Faculty of Medicine, The Hebrew University of Jerusalem, conforming to the standards for care and use of laboratory animals of the Hebrew University of Jerusalem. Male rats of Sabra strain weighing 350-420g were used. The animals were fed standard laboratory chow and tap water ad libitum. All *in vivo* experiments were conducted

under general anaesthesia achieved with 80 mg/kg ketamine and 5mg/kg xylazine administered IP.

Rat carotid catheter injury model

The distal left common and external carotid arteries were exposed through a midline incision in the neck. The left common carotid artery was denuded of endothelium by the intraluminal passage of a 2F balloon catheter introduced through the external carotid artery. The catheter was passed three times with the balloon distended sufficiently with saline to generate a slight resistance. The catheter was then removed and the external carotid artery was ligated, and the wound was closed with surgical staples.

Seven rats served as the control group, and 6 rats as the treated group (randomly chosen). Liposomal clodronate was injected IV to the "treated group" one day prior to the arterial injury (6 mg of clodronate per rat) and repeated on day 6. In the control group similar injections were administered but with "empty" or blank liposomes (no clodronate).

All animals were sacrificed 14 days after injury by an overdose of pentobarbital. Arteries were perfusion-fixed with 150 ml of 4% formaldehyde solution pH 7.4 at 100 mm Hg. The right atrium was dissected and an 18G catheter connected to the perfusion system was inserted in the left ventricle. The arterial segments were dissected, cut, gently separated from the polymer, and postfixed for at least 48 hours in the same fixative solution. The arterial segments were embedded in paraffin and cut at 8-10 sites 600 μm apart. Sections of 6 μm were then mounted and stained with Verhoeff's elastin stain for histologic examination.

Morphometric analysis

The slides were examined microscopically by an investigator blinded to the type of the experimental group. Six to eight sections in each slide were evaluated by computerized morphometric analysis and the averaged section data were further used as a representative of a whole slide for comparisons between groups. The residual lumen, the area bounded by the internal elastic lamina (original lumen), and the area circumscribed by the external elastic lamina ("total arterial area") were

measured directly. The degree of neointimal thickening was expressed as the ratio between the area of the neointimal and the original lumen (% stenosis), and as the ratio between the neointimal area to the area of the media (N/M). The medial area, an indirect index of SMC viability, was determined as the difference between the total arterial area and the original lumen area.

The surgical procedure and treatment did not cause mortality or apparent morbidity of the animals.

As seen in Fig. 1 the ratio between the neointimal area to the area of the media (N/M) was significantly reduced following treatment with clodronate-encapsulated in liposomes. The N/M ratio in clodronate treated rats was 0.28 ± 0.23 as compared to 1.42 ± 0.26 in the control group (mean ± SD, $p < 0.01$). Similarly as seen in Fig. 2, significant inhibition of % stenosis was achieved in the treated group: 9.8 ± 7.76 vs. 41.53 ± 7.9, treated and control groups, respectively (mean ± SD, $p < 0.01$). There were no apparent systemic side effects nor any effects on somatic growth as illustrated in Fig. 3.

Thus, the results of the experiments indicate that treatment of rats with clodronate-containing liposomes significantly reduces restenosis observed as neointimal formation following balloon-injury of the carotid artery.

EXAMPLE 2

The antirestenotic effects of liposomal clodronate injections were studied in the balloon-injured rat and hypercholesterolemic rabbit carotid arterial models. The rats were treated by clodronate-containing liposomes, empty liposomes (control), and clodronate in solution (additional control). The dose of clodronate injected was 1.5 and 15 mg/kg administered one day before procedure (-1) and/or on day 6 (+6) post injury. The rabbits (following 30 days of atherosclerotic diet) were treated one day prior to balloon angioplasty by liposomal clodronate (10 mg/kg). The lumen, neointimal, medial and vessel areas and volumes were measured in the treated and control animal groups by digital planimetry of histological sections, at 14 and 30 days post injury in the rat and rabbit models, respectively.

The results of the antirestenotic effects of liposomal clodronate are shown in Figure 4. As illustrated, no significant differences were found between treatments

with empty liposomes, and free clodronate in solution, which both exhibited marked neointimal formation. The extent of mean neointimal formation, mean neointimal to media ratio (N/M), and % stenosis following treatment with clodronate-laden liposomes was significantly reduced. However, the medial area was not affected by the various treatments indicating no deleterious effects on quiescent cells. Moreover, there were neither apparent systemic side effects nor any effects on bone and somatic growth. Significantly, more potent treatments were evaluated, specifically, 1 x 15 mg/kg (-1) and 2 x 15 mg/kg (-1, and +6) injections, with no significant difference between them. Similar findings of no adverse effects were also observed in the rabbits' study. Liposomal clodronate was significantly effective in reducing neointimal formation and % stenosis.

Furthermore, injection of silica particles also reduces intimal formation (Fig. 4). This observation can be attributed to the known inhibiting effect of silica on macrophages.

The results of the experiment indicated that treatment by clodronate-containing liposomes significantly reduces neointimal formation following balloon-injury both in rat and rabbit models. There were neither apparent systemic and local side effects nor any effects on somatic growth. It should be noted that although BPs are known as affecting bone, no effects on the bone or on calcium and phosphorus levels in bone and blood were observed following treatment with liposomal preparation of clodronate.

EXAMPLE 3: Effect of ISA Composites on RAW 264 Proliferation

ISA Encapsulated in Nanoparticles

Nanoparticles (NP) were prepared by a novel solvent evaporation polymer precipitation technique using a double emulsion system. 20 mg of ISA acid (Cohen, H. et al., 1999, Pharm. Res., 16: 1399-406) and 8.9 mg of $NaHCO_3$ were dissolved in 0.5 ml Tris buffer, and 90 mg of PLGA were dissolved in 3 ml dichloromethane. The aqueous sodium ISA solution was added to the PLGA organic solution and a water in oil (W/O) emulsion was formed by sonication over an ice-bath using a probe type sonicator. This W/O emulsion was then added to a 2% polyvinyl alcohol

(PVA) (20 ml) filter sterilized solution, and the pH was adjusted to 7.4 with NaOH solution containing $CaCl_2$ in a molar ratio of 2:1 to ISA. The mixture was mixed over an ice bath, forming a double emulsion (W/O/W). The emulsion was stirred at 4°C overnight to allow evaporation of the organic solvent.

Nanoparticles which did not have any drugs encapsulated within (termed blank NP) were prepared according to the same procedure by omitting the drug. Ca^{+2}-ISA salt with PVA and Ca^{+2}-ISA salt were prepared according to the same procedure by omitting the polymer or by omitting the polymer and the PVA, respectively. Ca^{+2} –ISA was adsorbed on blank nanoparticles (prepared as above) by dispersing the nanoparticles in the buffer and precipitating ISA-calcium with the same ingredients as used above. The amount of drug entrapped in the NPs was determined spectrophotometrically following sequential ultracentrifugation.

The influence of various formulation parameters on drug entrapment efficiency, release properties, and size have been examined. For example, the following parameters were evaluated: buffers, emulsifiers, various amounts of ISA, $CaCl_2$ (including without), amount of PVA (including without), different amounts and molecular weights of PLGA/PLA polymers, temperature, yield and extrament efficiency. The various formulation steps resulted in the development of spherical nanoparticles containing ISA. Furthermore, NP formulation reproducibility was successfully demonstrated. High yield, entrapment efficiency, as well as lyophilizability are important features for any nanoparticulate carrier.

Figure 5 tabulates typical formulations of both ISA loaded nanoparticles and blank nanoparticles. Whereas Figure 5 illustrates a typical formulation, it shall be understood that additional formulations may also be effective. The formulation parameters tabulated include the size of the nanoparticle both before and after lyophilization, the percent recovery, the ISA entrapment (measuered as % of initial), the final ISA content, the ISA in NP, the ISA in the supernatant, the initial ISA content, the Ca^{+2} recovery, the Ca^{+2} in the supernatant, the Ca^{+2} in NP, the ratio of Ca^{+2} to ISA, and the Zeta potential. The size of the nanoparticle before lyophilization was 376 nm and increased 396 nm after lyophilization. Typically, the size of the nanoparticles range from 100-500 nm, depending not only upon

preparation and sterilization techniques, but also upon the mode of administration. Lyophilization not only increases the shelf-life of the nanoparticles, but also enables sterilization of the NP through γ irradiation. The percent recovery is tabulated in Figure 5 as 68.7%. Essential to the antirestenotic effect of the bisphosphonate NP is the bisphosphonate content within the nanoparticle and the entrapment efficiency. These parameters are measured by: ISA entrapment, tabulated in Figure 5 as 59.6%; final ISA content, measured as the ratio between ISA weight over the NP total weight, and tabulated as 16.5%, but may range from 25 to 40%; the amount of ISA in NP, tabulated as 11.9 mg, but may be changed accordingly; the amount of ISA in the supernatant tabulated as 1.1 mg, but may range as a function of the entrapment efficiency mentioned above; and the initial ISA content, tabulated as 18.1%, but may be any percentage in an appropriate ratio to the polymer amount. In order to reduce the solubility of the hydrophilic drug and increase its entrapment efficiency, a cation, such as Ca^{+2} is added to the composite. The parameters associated with Ca^{+2} include: Ca^{+2} recovery, tabulated as 72.3%; Ca^{+2} in supernatant, tabulated as 38%; Ca^{+2} in NP, tabulated as 34%; and Ca^{+2}/ISA, tabulated as 1.2 mols. However, these parameters may range as a function of the ratio between calcium to ISA, the type of additives, the pH of solution and other like factors. Additionally, the Zeta potential is tabulated as –5.7. A negative value for the nanoparticle zeta potential is important for efficient uptake by phagocytic cells (e.g. macrophages).

Furthermore, lyophilized NPs were shown to have similar properties to non-lyophilized NPs, in both in vitro and in vivo experiments. Indeed, this is of significant importance since NP sterilization could be obtained through γ irradiation of dry NP, ethylene-oxide sterilization, steam sterilization (when other polymers are used) or filter sterilization.

Each particle carrier (e.g., polymeric micro/nanoparticle) exhibited a certain entrapment efficiency for the bisphosphonate drug. The ISA entrapment efficiency in the NP reached 60% and is substantially higher than any efficiency that is reported in the literature for any given hydrophilic drug in either PLGA nanoparticles or liposomes.

The bisphosphonate, ISA, serves as a model bisphosphonate. The physicochemical properties of other BPs are similar to those of ISA. Moreover, ISA serves as model drug for a low molecular weight, hydrophilic, charged molecule and, as such, was used as a model bisphosphonate in the experiments herein. For properties of ISA, refer to, Cohen H, Alferiev IS, Monkkonen J, Seibel MJ, Pinto T, Ezra A, Solomon V, Stepensky D, Sagi H, Ornoy A, Patlas N, Hagele G, Hoffman A, Breuer E, Golomb G, 1999, "Synthesis and preclinical pharmacology of 2-(2-aminopyrimidinio) ethylidene-1,1-bisphosphonic acid betaine (ISA-13-1)-a novel bisphosphonate." Pharm Res.;16: 1399-406.

In vitro bioactivity

The effect of ISA on the growth of RAW 264 cells was determined. RAW 264 cells, which are derived from the murine macrophage cell lines, were plated at $2x10^4$ cells per well in 24-well plates and allowed to grow for approximately 24 hours in DMEM. The cells were then treated with various compositions of the ISA drug, specifically, free ISA, the ISA-Ca^{+2} salt, and ISA encapsulated in a nanoparticle ("ISA NP"). As discussed supra, the ISA NP contains Ca^{+2} to lower the solubility of the bisphosphonate and increase its entrapment efficiency. For comparison purposes, the RAW 264 cells were also treated with blank NP, i.e., nanoparticles with no drug embedded therein. The cells were then analyzed 48 hours after treatment. Analysis included cell counting by Coulter counter and cell viability by tryphan blue exclusion assay.

The effect of ISA compositions on the growth of RAW 264 cells in vitro is illustrated in Figure 6. The cell proliferation in buffer only was termed as 100%. The data represented is the mean with a ± SD ($6 \leq N \leq 27$). As the legend designates, *$P<0.05$, **$P<0.01$ in comparison to buffer and \$ $P<0.01$ in comparison to ISA+Ca^{2} indicating that the differences are statistically significant.

As depicted in Figure 6, free ISA had only a minor effect on the growth of RAW 264 cells (macrophages). However, the addition of equal Ca^{+2} concentrations to ISA to form a salt, potentiated the bisphosphonates activity, and significantly suppressed the proliferation of RAW 264 cells in a dose response manner. Indeed, the use of ISA-Ca^{+2} salt served as an appropriate control group to ISA NP activities

since both compositions contained equivalent molar amounts of Ca^{+2} and ISA. ISA encapsulated in nanoparticles ("ISA NP") were found to be potent inhibitors of the growth of RAW 264 cells. As illustrated in Figure 6, the ISA NP were far more potent than both ISA and ISA + Ca^{+2}. The blank NP had no effect on the proliferation of RAW 264 cells up to 100 μM, indicating that the ISA NP growth inhibitory effect was caused by ISA and not by the polymer.

Additionally, the effect of Ca^{+2}-ISA salt formulations on RAW 264 proliferation is presented in Figure 7. The following formulations were evaluated: Ca^{+2}-ISA salt, Ca^{+2}-ISA salt + PVA, and Ca^{+2}-ISA adsorbed on the surface of a blank NP. The cell proliferation in the buffer only was termed as 100%. N.B., a lower column represents higher potency.

As depicted in Figure 7, Ca^{+2}-ISA salt potently inhibited the proliferation of RAW 264 cells at 50 μM, and the addition of PVA to Ca^{+2}-ISA salt further potentiated its activity. As discussed *supra*, PVA is an additive which is used to stabilize the BP-salt/complex. However, Ca^{+2}-ISA adsorbed on the surface of the blank NP significantly suppressed RAW 264 cell proliferation. As illustrated in Figures 6 and 7, the Ca^{+2}-ISA salt (50μM) adsorbed on a blank NP and ISA encapsulated in NP (50 μM) were comparable inhibitors of RAW 264 proliferation. In summary, the results of the experiment indicate that Ca^{+2}-ISA salt particulates are useful in inhibiting restenosis by eliminating or inhibiting macrophages.

However, in vivo utilization of Ca^{+2}-ISA salt particulates to eliminate macrophages, requires maintaining the composites in the nanometer size in order to be suitable for IV use. Maintaining the salt composites in the nanometer size may be achieved with a proper surfactant during the Ca^{+2}-ISA salt preparation. Indeed, the benefit of utilizing a Ca^{+2}-ISA salt composite to treat restenosis is its simplicity and the avoidance of PLGA use. However, a possible drawback of this approach might be the rapid dissolution of the Ca^{+2}-ISA salt seconds after administration, due to high dilution.

Similar experiments were conducted to determine the effect of ISA NP on the proliferation of smooth muscle cells extracted from the aortas of adult male Sabra rats and 3T3 fibroblast cells. The results of the experiment (not shown)

indicate that ISA NP significantly inhibited the growth of rat SMC and 3T3 cells (fibroblasts).

In summary, ISA encapsulated within NP was found to inhibit the growth of the three main cell types involved in the restenotic cascade, namely macrophages (RAW 264 cells), fibroblasts (3T3 cells) and smooth-muscle cells (SMC).

EXAMPLE 4: Effect of ISA-Nanoparticles Rat Carotid Model of Restenosis

In vivo bioactivity

The following experiment examined the effect of ISA encapsulated in NP, specifically PLGA based NP, on neointimal formation. The ISA NP were prepared as described *supra*, in Example 3. Additionally, Male Sabra rats were used and prepared according to the rat carotid catheter injury model described *supra*, in Example 1. ISA NP was injected IV to the "treated group" one day prior to the arterial injury (-1d) at a dosage of 15 mg/kg. In the control group, similar injections were administered but with blank NP, i.e. nanoparticles with no ISA encapsulated or adsorbed therein.

The animals were then sacrificed 14 days of injury, their arterial segments dissected and prepared for histologic examination. (Refer *supra*, in Example 1, for details). The arterial segments were evaluated by computerized morphometric analysis. The residual lumen, the area bounded by the internal elastic lamina (original lumen), and the area circumscribed by the external elastic lamina (total arterial area) were measured directly. The degree of neointimal thickening was expressed as the ratio between the area of the neointimal and the original lumen (% stenosis), and as the ratio between the neointimal area to the area of the media (N/M). The medial area, an indirect index of SMC viability, was determined as the difference between the total arterial area and the original lumen area.

The experimental results indicate that administration of ISA NP significantly inhibited vascular neointimal formation in comparison to blank NP treatment. As indicated in Figure 8A, significant inhibition of % stenosis was achieved in the ISA NP treated group (n=12). Similarly, as illustrated in Figure 8B, the extent of mean neointimal formation and mean neointimal to media ratio (N/M) following treatment

with ISA NP was significantly reduced. Thus, the experimental results indicate that treatment of rats with ISA encapsulated nanoparticles via IV administration significantly reduces restenosis observed as neointimal formation following balloon-injury of the carotid artery.

Additionally, SC administration of ISA encapsulated in nanoparticles was evaluated and found to significantly inhibit neointimal formation 14 days after vascular injury. However, the SC administration provided a weaker restenosis inhibiting effect than that obtained from IV delivery of the ISA NP.

In summary, the above experiments indicate that the improved stability, high drug entrapment efficiency, and increased bioactivity of ISA encapsulated in NP or ISA absorbed on NP, possess novel and important advantages for clinical applications. Additionally, Ca-BP salt particulates were also found to be bioactive and effective in inhibiting proliferation of monocytes.

EXAMPLE 5: Effect of Alendronate-Nanoparticles in Rabbit Model of Restenosis

Alendronate NPs

Alendronate encapsulated within nanoparticles ("alendronate NPs") were prepared by a novel solvent evaporation polymer precipitation technique using a double emulsion system. 20 mg of alendronate were dissolved in 0.5 ml Tris buffer with 2.8% PVA and 90 mg of PLGA were dissolved in 3 ml dichloromethane. The aqueous alendronate solution was added to PLGA organic solution and a water in oil (W/O) emulsion was formed by sonication over an ice bath using a probe type sonicator, at 14 W for 90 seconds. This W/O emulsion was further added to 10.5 ml of Tris buffer (containing 2% PVA and $CaCl_2$ solution in molar ratio 2:1 to alendronate), and sonicated for 90 seconds over an ice bath, forming the double emulsion (W/O/W). The emulsion was stirred at 4^0C for 3 hours, to allow evaporation of the organic solvent.

Figure 9 tabulates the formulation parameters for the alendronate NP formed. Although Figure 9 tabulates a typical alendronate NP, it shall be understood that various formulations may also be effective. The formulation parameters include

size, alendronate entrapment, the amount of alendronate in NP, the alendronate in supernatant, the initial amount of alendronate, the PLGA amount and the volume of a 0.246M calcium chloride solution. The size of the nanoparticle was 219 nm. As discussed *supra*, typically, the size of the nanoparticles range from 100-500 nm, depending not only upon preparation and sterilization techniques, but also upon the mode of administration. Essential to the antirestenotic effect of the bisphosphonate NP is the bisphosphonate content within the nanoparticle and the entrapment efficiency. These parameters are measured by: alendronate entrapment, tabulated in Figure 9 as 55.1%; alendronate in NP, tabulated as 1.002 mg/ml; alendronate in the supernatant, tabulated as 0.232 mg/ml; the initial amount of alendronate, tabulated as 20 mg; the PLGA amount, tabulated as 90 mg; and the volume of calcium chloride, tabulated as 0.5 ml. However, these parameters may be modified to provide additional formulations. In the experiment, the effect of alendronate NP on RAW 264 proliferation was evaluated. For procedures describing the growth of RAW 264 cells and subsequent treatment with NP refer to Example 3, *supra*. As depicted in Figure 10, alendronate NP are potent inhibitors of macrophages, whose activity increases with concentration.

Balloon-injured hypercholesterolemic rabbit arterial model

The antirestenotic effect of alendronate NPs was evaluated in both the balloon-injured rat and the balloon-injured hypercholesterolemic rabbit carotid arterial models. While alendronate NPs were successful in reducing restenosis in both models, of significant importance is the marked efficacy discovered in the hypercholesterolemic model of balloon-injured rabbits. The rabbits were treated by alendronate NPs and blank NPs (control) via SC and IV administration in order to compare the differences between the two modes of administration. A group of rabbits were treated one day prior to balloon angioplasty with 1.5 mg/kg of alendronate NPs via SC administration. For comparison purposes, two additional groups were also treated one day before the procedure (-1d) with 0.15 mg/kg of alendronate NPs via SC and IV administration. The lumen, neointimal, medial and vessel areas were measured in the treated and control rabbit groups by digital planimetry of histological sections at 30 days post injury.

The results of the antirestenotic effects of alendronate NPs in the balloon-injured hypercholesterolemic rabbit model are illustrated in Figures 11, 12a and 12b.

Figure 11 illustrates that the % restenosis following treatment with alendronate NPs via SC administration was significantly reduced.

Figures 12a and 12b compare the effect of two dosage amounts of the alendronate NPs via SC administration, specifically 1.5 and 0.15 mg/kg and also the effects of two modes of administration, IV and SC. As illustrated in the figures, the mean neointimal to media ratio (N/M) and % stenosis was reduced in a dose response manner. Specifically, a dose of 1.5 mg/kg was more effective in reducing N/M and % restenosis in comparison to 0.15 mg/kg. Moreover, the inhibition of neointimal formation and % restenosis by alendronate NPs via SC administration was slightly greater than that obtained via IV delivery, although not significant in light of the experimental standard deviation. Additionally, there were neither apparent systemic side effects nor any effects on bone and somatic growth.

In conclusion, alendronate NP is a highly potent inhibitor of restenosis in animal models via SC and IV administration. Furthermore, the dosage range of 0.15 to 1.5 mg/kg was found to be the most potent delivery system in preventing restenosis.

EXAMPLE 6: Effect of Alendronate-Nanoparticles in Human Blood

In this example, the ability of alendronate NPs to decrease the number of monocytes in human blood was studied. Human blood was drawn to EDTA-containing test tubes and 200 μl were incubated for 24h in 37^{0}C on a shaker with the indicated doses of alendronate-nanoparticles in 50 μl diluted in 50 of PBS. Control samples were incubated with 50 μl. The samples were then incubated (30 min. 4^{0}C, in the dark) with RPE-conjugated anti-CD14 Ab (specific for monocytes) for 30 min. RBC (red blood cells) were lysed by FACS lysing solution (Becton-Dickinson, San-Jose, CA) and distilled water, and following washings in FACS (fluorescence activated cell sorting) solution, flow cytometry analysis was performed. Monocytes were detected by side-scattering and fluorescence.

The results of the anti-proliferative effects of alendronate-nanoparticles on monocytes in human blood are illustrated in Figure 13. It is apparent that alendronate NPs potently decreased the amount of monocytes in human blood in a dose response manner. Since monocytes, in their normal state, are recruited to the areas traumatized by angioplasty or other intrusive intervention and initiate the proliferation of smooth-muscle cells, thus leading to restenosis, inhibiting the number of monocytes will inhibit restenosis.

EXAMPLE 7 – Effect of Liposomal clodronate on IL-1β production and transcription and MMP-2 activity

The effects of liposomal clodronate on interleukin 1-β (IL-1β) production and transcription and matrix metalloproteinase-2 (MMP-2) activity were studied in the balloon-injured rat and the hypercholesterolemic rabbit carotid arterial models. A group of male Sabra Rats was prepared according to the rat carotid catheter injury model described *supra*, in Example 1. The hypercholesterolemic rabbit model consisted of New Zealand White rabbits weighing 2.5-3.5 kg. The rabbits were fed an atherogenic diet of 2% cholesterol and 6% peanut oil starting 30 days before angioplasty and hypercholesterolemia was established (plasma cholesterol > 1,200 mg/dL). The rabbits were then anesthesized by xylazine (7 mg/kg) and ketamine (40 mg/kg). Heparin (200 units/kg), atropine (0.05 mg) and norfloxacin nicotinate (70 mg) were also administered to the rabbits. Thereafter, balloon injury was performed on the left common carotid artery with a 3 mm angioplasty balloon catheter (Cordis, Miami, FL, USA, 2 x 1 min inflation at 8 atm). In both the rabbit and rat models, liposomal clodronate (LC) was injected IV to the "treated group" both one day prior to the arterial injury (-1d) and six days after (+6) at a dosage of 15 mg/kg. The control animals were treated with empty liposomes, i.e., liposomes with no bisphosphonates encapsulated therein.

IL-1β production and transcription

Arteries and livers were homogenized in collagenase buffer (5 mM $CaCl_2$, 50 mM Tris, 0.02% Brij 35, 0.2 M NaCl, pH 7.6). IL-1β was measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). For RT-PCR

analysis, RNA from the carotid arteries was extracted using a RNA isolation kit. (Life Technologies Inc., USA). Quality, size and quantity of RNA were examined by conventional 1.0% agarose gel electrophoresis (Sigma) and spectrophotometry. Total RNA (2 μg) was used for the synthesis of first strand cDNA using Superscript reverse transcriptase and a mixture (1:1) of oligo (dT) and random hexanucleotides in 20 ml reaction volume. First strand cDNA was amplified by PCR. To ensure the quality of the RNA preparation and to normalize the RT-PCR protocol, β-actin RT-PCR products were also produced for all samples. cDNA (2 μl) was added to a 50 ml reaction mixture containing 5 μl 10*PCR reaction buffer, 2.0 mM $MgCl_2$, 20 mM of each dATP, dCTP, dGTP, and dTTP, 200 nM of each oligonucleotide primer, and 1.0 unit Taq DNA polymerase. Oligonucleotide primers for rabbit IL-1β and β-actin were synthesized based on the following nucleotide sequences: IL-1β sense primer 5'-TAC AAC AAGAGC TTC CGG CA (SEQ. ID. NO. 1); IL-1beta antisense primer 5'-GGC CAC AGG TAT CTT GTC GT (SEQ. ID. NO. 2); β-actin sense primer 5'-ACG TTC AAC ACG CCG GCC AT (SEQ. ID. NO. 3); β-actin antisense primer 5'-GGA TGT CCA CGT CGC ACT TC (SEQ. ID. NO. 4). Amplification was performed using a DNA thermal cycler for 37 cycles, where a cycle profile consisted of 1 minute at 94°C for denaturation, 1 minute at 55° for annealing, and 1 minute at 72° for extension. The size of amplified fragments was 354 and 493 bp for IL-1β and β-actin, respectively. Electrophoresis of 10 μl of the reaction mixture on a 1.5% agarose gel containing ethidium bromide was performed to evaluate amplification and size of generated fragments. PCR marker (Promega, USA) was used as a standard size marker. The bands' intensity was quantified by densitometry, and values of the bands were normalized to β-actin mRNA expression.

MMP-2 activity

The supernatant of arteries homogenate in collagenase buffer (see above) was analyzed for collagenase activity. Samples of arteries were separated on gelatin-impregnated (1 mg/ml: Difco, Detroit, MI, USA) SDS 8% polyacrylamide gels under non-reducing conditions, followed by 30 minutes of shaking in 2.5% Triton

X-100 (BDH, Poole, UK). The gels were incubated for 16 hours at 37°C in a collagenase buffer, and stained with 0.5% Coomassie G-250 (BioRad, Richmond, CA) in methanol/acetic acid/H_2O (30:10:60). Band intensity was determined by computerized densitometry (Molecular Dynamics type 300A).

As illustrated in Figure 14A, analysis of IL-1β levels in rat arterial tissue following balloon injury (control animals) revealed a bell shape pattern peaking at 6 days following injury (37.3 ± 9.6 pg/mg protein) and returning to basal levels after 30 days. However, a significant decrease of IL-1β levels was observed on days 3 and 6 post-injury, following LC-treatment. As illustrated in Figure 14B, a similar response was observed in the rabbit artery, with a significant decrease of IL-1β levels on days 2, 4 and 6 post-injury, following LC-treatment.

The reduction in arterial IL-1β levels following LC-treatment was associated with a marked decrease in IL-1β transcription. IL-1β mRNA transcription in rabbits' arteries was analyzed following LC-treatment, which was administered the day before balloon injury (-1). The gel electrophoresis of the resultant reaction mixture following RT-PCR analysis is illustrated in Figure 15. The RT-PCR analysis illustrates that in control animals (no treatment with LC), IL-1β mRNA transcription was stronger three days after the injury (+3) than one day after the injury (+1). However, IL-1β transcription on both day one (+1) and day three (+3) after the injury, was significantly reduced by LC treatment. In Figure 15, Lane 1 represents PCR markers (50, 150, 300, 500, 750, 1000 bp); lanes 2 and 3 represent LC-treated and untreated (control), on day +1, respectively; and lanes 4 and 5 represent LC-treated and untreated (control), on day +3, respectively. Note the strong signal (at 354 bp) of IL-1β mRNA expression in untreated (control) animals (lanes 2 and 4) that was suppressed by LC treatment (lanes 3 and 5). Expression of β–actin mRNA expression (493 bp) was used as loading control in the same samples (lower panel). IL-1β mRNA levels (densitometry analysis relative to β–actin mRNA) were found to be 0.45±0.24 and 0.37±0.44 on day +1, 0.59±0.2 and 0.12 ±0.1 on day +3, LC-treated and untreated animals, respectively (3 independent RT-PCR reactions).

Additionally, IL-1β levels in the liver were also examined. A significant reduction was noted after a single injection of LC on day -1 inclining to basal levels at 30 days (data not shown).

As illustrated in Figure 14c, MMP-2 activity in rats' arterial tissue increased following injury, exhibiting a bell shape pattern peaking at 14 days (252 ± 12 and 402 ± 44, at 6 and 14 days, respectively), and returning to basal levels at 30 days. However, treatment with LC resulted in a significant reduction of MMP-2 activity at 6 and 14 days (152 ± 23 and 284 ± 17, respectively). Similarly, in the rabbit's artery, the surge of MMP-2 activity was less than that of the rat's artery, but the effect of LC-treatment was more pronounced (See, Figure 14d). As illustrated in Figure 14d, MMP-2 activity at 6 days was 248 ± 42 and 52 ± 5, in control and LC-treated rabbits, respectively, returning to the baseline approximately 14 days after injury.

EXAMPLE 8 – Effect of Liposomal clodronate on PDGF-BB, PDGFβR and PDGFβR tyrosine phosphorylation

The effect of liposomal clodronate on PDGF-BB, platelet-derived growth factor β receptor (PDGFβR) and PDGFβR tyrosine phosphorylation was studied in the balloon-injured rat arterial model. A group of male Sabra Rats was prepared according to the rat carotid catheter injury model described *supra*, in Example 1. Carotid arteries were rapidly retrieved before injury and at day 14, rinsed in cold PBS and immediately deep-frozen (-70 °C) until further processing. Frozen segments were mechanically minced on dry ice. There was a total of 12 animals in each group, with four arteries being pooled for each run. Liposomal clodronate (LC) was injected IV to the "treated group" one day prior to the arterial injury (-1d) and six days later (+6) at a dosage of 15 mg/kg. Proteins were extracted using lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Triton X-100, 10 mM EDTA, 1 mM PMSF, 100 μM sodium orthovanadate and 1% aprotinin) and pooled for 4 animals in each group. Protein content was determined using a modified Lowry protocol, and samples of 100 μg were subjected to SDS-PAGE (7.5% or 12%) and blotted onto a nitrocellulose membrane (Hybond C extra, Amersham) of PVDF membrane (Roth) for the analysis of PDGFβR or PDGF-B chain, respectively. PDGFβR

protein was detected using a polyclonal antibody (SC-431, Santa Cruz, USA) and a polyclonal alkaline phosphatase-conjugated goat anti rabbit antiserum (Tropix, USA), and tyrosine phosphorylated proteins were detected using a mixture of monoclonal antibodies PY20 (Transduction Laboratories, USA) and 4G10 (UBI, USA) followed by the application of a chemoluminescence-based detection system including a polyclonal alkaline phosphatase-conjugated anti-mouse antiserum (CDP Star, TROPIX). PDGF-BB protein was detected using the monoclonal antibody PGF007 (Mochida), a horseradish-conjugated rabbit anti-mouse antiserum (DAKO) and the detection system Super Signal Ultra (Pierce, Germany). Quantification of the data was made by means of LAS-1000 Imager (Fuji, Japan).

As illustrated in Figure 16a, the activation of PDGFβR (i.e., tyrosine phosphorylation) markedly increased to 135% of the baseline levels in the balloon-injured artery of untreated rats, while it was barely detectable in LC treated rats, i.e. below baseline activity. Note the band representing activated PDGFβR at 190 kDa. Additionally, as depicted in Figure 16b, vascular injury resulted in the upregulation of PDGFβR protein both in the untreated group (121%) and the LC-treated group (233%). Injury resulted in a strong accumulation of PDGF-B protein within the vessel wall on days 1 and 3 after injury (46 kDa), reaching 333% and 219% of the baseline level, respectively (Fig. 16c). Further illustrated in Figure 16c is that in LC-treated rats, this accumulation of PDGF-B was strongly reduced (181% and 168%, on days 1 and 3, respectively), in correlation with the reduced activation of PDGFβR at these time points.

The results of the experiments described *supra*, clearly indicate that treatment of rats and rabbits with liposomal clodronate significantly reduces the transcription and production of interleukin 1-β, the activity of matrix metalloproteinase-2, the activation of platelet-derived growth factor β receptor (PDGFβR), and the levels of PDGF-B protein.

The contents of all patents, published articles, books, reference manuals, and abstracts, as cited herein are hereby incorporated by reference in their entirety to more fully describe the state of the art to which the invention pertains.

As various changes can be made in the above-described subject matter without departing from the scope and spirit of the present invention, it is intended that all subject matter contained in the above description, or defined in the appended claims, be interpreted as descriptive and illustrative of the present invention. Many modifications and variations of the present invention are possible in light of the above teachings.

WE CLAIM:

1. A method of treating or preventing restenosis, comprising:

administering to an individual an effective amount of a compound, wherein the compound inhibits or depletes blood monocytes or tissue macrophages, thereby inhibiting restenosis.

2. A method of inhibiting or depleting macrophages or monocytes for treating or preventing restenosis, comprising:

administering to an individual an effective amount of a formulation comprising an encapsulated compound, thereby inhibiting restenosis.

3. A method of inhibiting or depleting macrophages or monocytes for treating or preventing restenosis, comprising:

administering to an individual an effective amount of a formulation comprising a particulate compound, thereby inhibiting restenosis.

4. A method of inhibiting the activity or production of cytokines or growth factors associated with vascular restenosis, comprising:

administering to an individual an effective amount of a formulation comprising an encapsulated compound, wherein the compound inhibits or depletes blood monocytes or tissue macrophages, thereby inhibiting restenosis.

5. A method of inhibiting the activity or production of cytokines or growth factors associated with vascular restenosis, comprising:

administering to an individual an effective amount of a formulation comprising a particulate compound, wherein the compound inhibits or depletes blood monocytes or tissue macrophages, thereby inhibiting restenosis.

6. The method as in one of claims 1-5, wherein the administering is intravenous, intra-arterial, intramuscular, subcutaneous, intraperitoneal, or delivered by a 'sweating balloon', a coated balloon or on a coated stent.

7. The method according to claim 6, wherein the formulation is administered systemically.

8. The method as in one of claims 1-5, wherein the formulation has a size range of 0.1-1.0 microns.

9. The method as in one of claims 1-5, wherein the compound is an intra-cellular toxin.

10. The method as in one of claims 1-5, wherein the compound is a bisphosphonate.

11. The method as in one of claims 1-5, wherein the compound is gallium.

12. The method according to claim 10, wherein the bisphosphonate is selected from the group consisting of clodronate, etidronate, tiludronate, pamidronate, alendronate, risendronate.

13. The method according to claim 2 or 4, wherein the compound is encapsulated in a particle selected from the group consisting of liposomes, polymeric particles, microparticles, nanoparticles, microspheres, and nanospheres.

14. The method according to claim 3 or 5, wherein particulates are selected from the group consisting of aggregates, flocculates, colloids, polymer chains, insoluble salts and insoluble complexes.

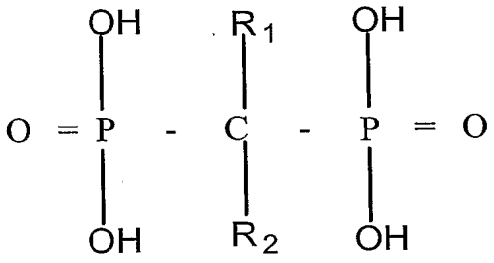
15. The method as in one of claims 2-5, wherein depletion of said monocytes and macrophages occurs through phagocytosis of the formulation.

16. The method according to claim 4 or 5, wherein the cytokines and growth factors are selected from the group consisting of interleukin 1-β, matrix metalloproteinase-2 (MMP-2), and platelet-derived growth factor β (PDGFβ).

17. The method according to claim 4 or 5, wherein the cytokines and growth factors are associated with cell types involved in the restenotic cascade.

18. The method according to claim 17, wherein the cells types include macrophages, monocytes, smooth-muscle cells, and fibroblasts.

19. The method as in one of claims 1-5, wherein said compound has the following formula (I):



(I)

wherein R_1 is H, OH or a halogen atom; and

R_2 is a halogen; linear or branched C_1-C_{10} alkyl or C_2-C_{10} alkenyl optionally substituted by heteroaryl or heterocyclyl C_1-C_{10} alkylamino or C_3-C_8 cycloalkylamino where the amino may be a primary, secondary or tertiary; -NHY where Y is hydrogen, C_3-C_8 cycloalkyl, aryl or heteroaryl; or R_2 is -SZ where Z is chlorosubstituted phenyl or pyridinyl.

20. The method according to claim 1, wherein the compound is administered before an angioplasty procedure.

21. The method according to claim 1, wherein the compound is administered the day of an angioplasty procedure.

22. The method according to claim 1, wherein the compound is administered after an angioplasty procedure.

23. The method as in one of claims 2-5, wherein the formulation is administered before an angioplasty procedure.

24. The method as in one of claims 2-5, wherein the formulation is administered the day of an angioplasty procedure.

25. The method as in one of claims 2-5, wherein the formulation is administered after an angioplasty procedure.

26. A method of inhibiting the activity or production of cytokines or growth factors associated with vascular restenosis comprising administering to an individual an effective amount of an encaspulated bisphosphonate, thereby inhibiting restenosis.

27. A method of inhibiting the activity or production of cytokines or growth factors associated with vascular restenosis comprising administering to an individual an effective amount of a particulate bisphosphonate, thereby inhibiting restenosis.

28. A pharmaceutical composition for the treatment or prevention of restenosis comprising a formulation selected from the group consisting of a compound, an encapsulated compound and a particulate compound, together with a pharmaceutically acceptable carrier, wherein the compound inhibits blood monocytes or tissue macrophages.

29. The pharmaceutical composition according to claim 28, in a dosage form suitable for intravenous, intra-arterial, intramuscular, subcutaneous, or intraperitoneal delivery.

30. The pharmaceutical composition according to claim 28, wherein the formulation is together with a diluent.

31. The pharmaceutical composition according to claim 28, wherein the formulation is together with a stabilizer.

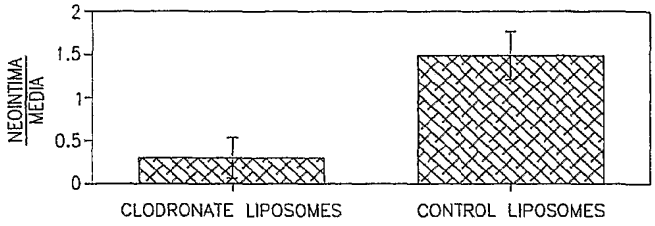


FIG.1

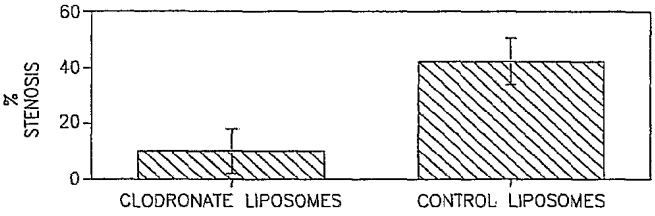


FIG.2

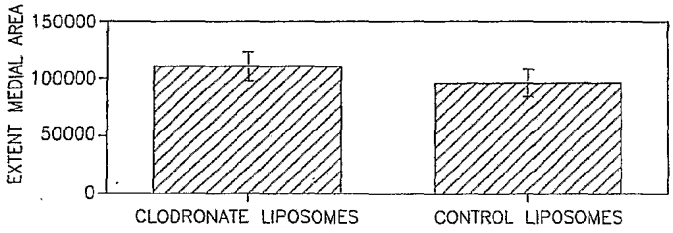


FIG.3

Treatment type	Animal (No.)	Dosing days	N/M	% stenosis
Empty liposomes	Rat (20)	-1, +6	1.62 ± 0.1	44.5 ± 2.0
Clodronate	Rat (10)	-1, +6	1.2 ± 0.2	40.2 ± 7.2
Liposomal clodronate 15 mg/kg	Rat (10)	-1	0.45 ± 0.1	15.4 ± 3.4
Liposomal clodronate 15 mg/kg	Rat (10)	-1, + 6	0.35 ± 0.07	12.0 ± 2.3
Empty liposomes	Rabbit (10)	-1	1.94 ± 0.3	86.6 ± 4.0
Liposomal clodronate 10 mg/kg	Rabbit (10)	-1	1.11 ± 0.2	71.5 ± 2.1
Silica particles 1000 mg/kg	Rat (10)	-1	0.8 ± 0.1	24.2 ± 3.8

FIG. 4

		Blank NP (N=12)	ISA NP (N=15)
Size (nm)	Before lyophilization	349±158	376±55
	After lyophilization	402±189	396± 70
Recovery (%) Final Wt./Initial Wt.		63.8±10.9	68.7±8.7
ISA entrapment (% of initial)			59.6±6.1
Final ISA content (%) (ISA weight/NP total weight)			16.5±2.7
ISA in NP (mg)			11.9±2.0
ISA in supernatant (mg)			1.1±0.45
Initial ISA content (%)			18.1
Ca^{2+} recovery (%)		88.3±10.9 (N=8)	72.3±11.9 (N=8)
Ca^{2+} in supernatant (%)		88	38
Ca^{+2} in NP (%)		0	34
Ca^{2+}/ISA (mol)			1.2±0.3
Zeta potential (mV)		-6.2±1.8	-5.7±0.9

FIG. 5

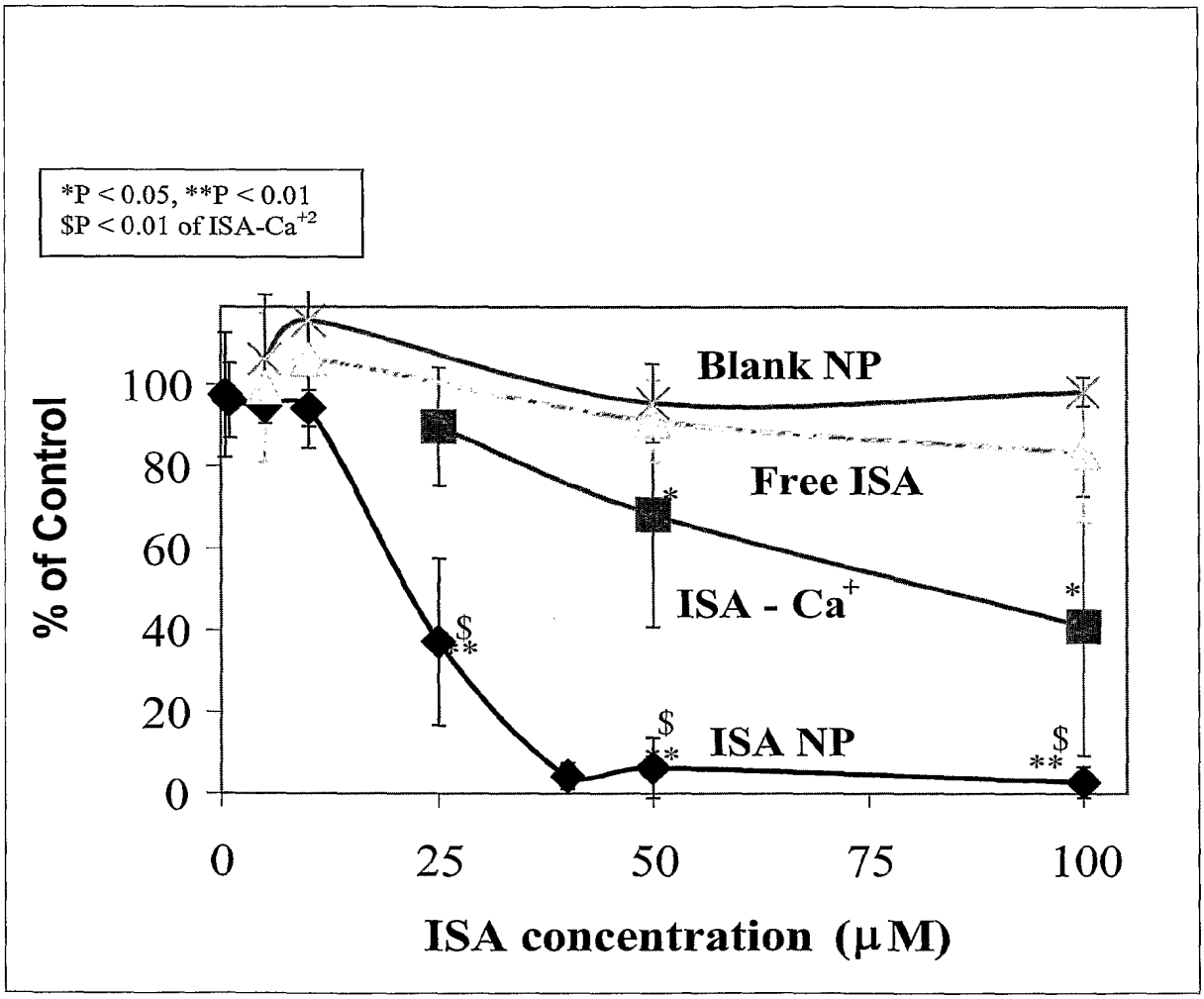

*P < 0.05, **P < 0.01
$P < 0.01 of ISA-Ca+2
% of Control
100
80
60
40
20
0
0
25
50
75
100
ISA concentration (μM)
Blank NP
Free ISA
ISA - Ca+
ISA NP

FIG. 6

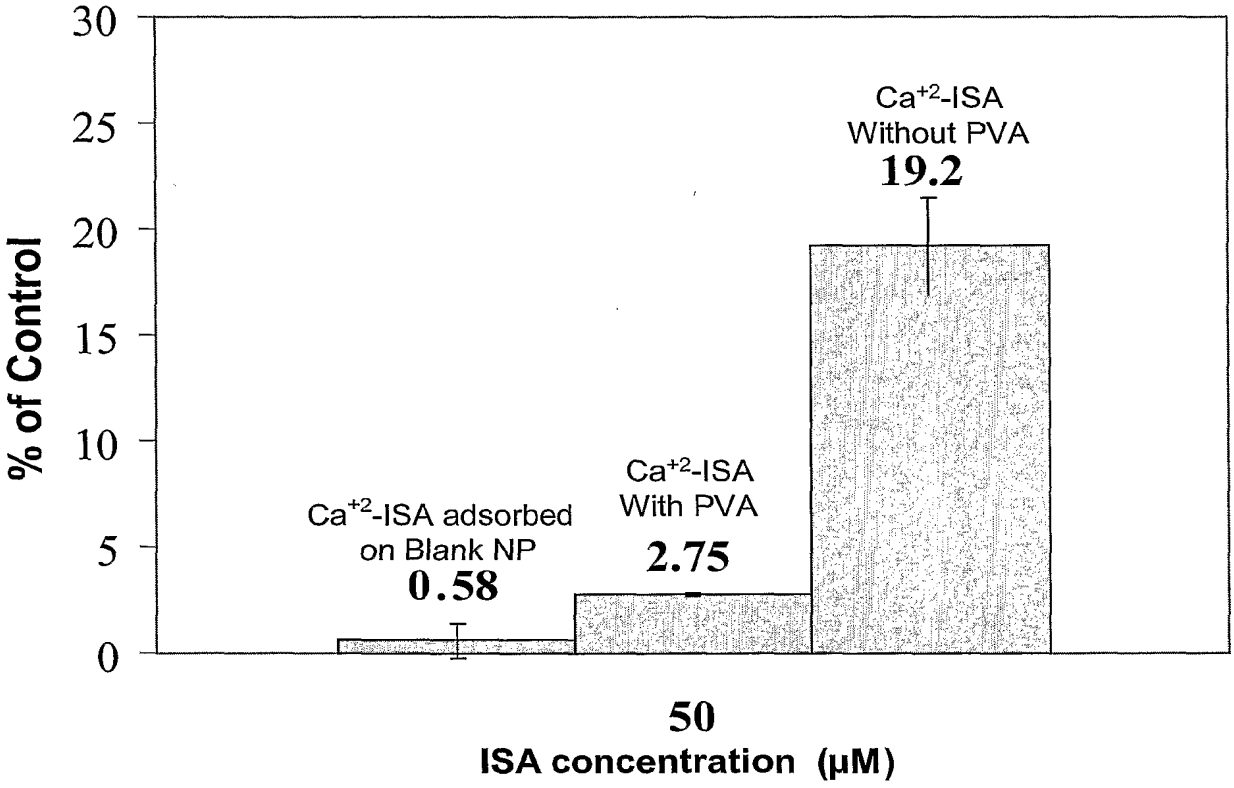

30
25
20
15
10
5
0
% of Control
Ca+2-ISA adsorbed on Blank NP
0.58
Ca+2-ISA With PVA
2.75
Ca+2-ISA Without PVA
19.2
50
ISA concentration (µM)

FIG. 7

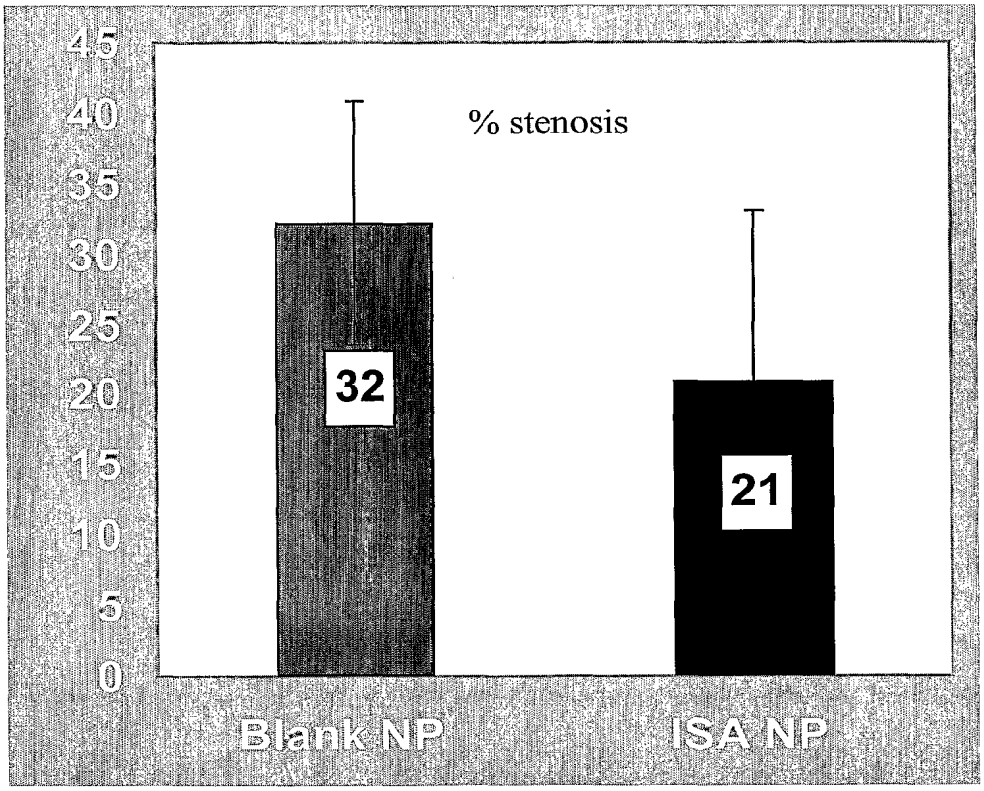


FIG. 8A

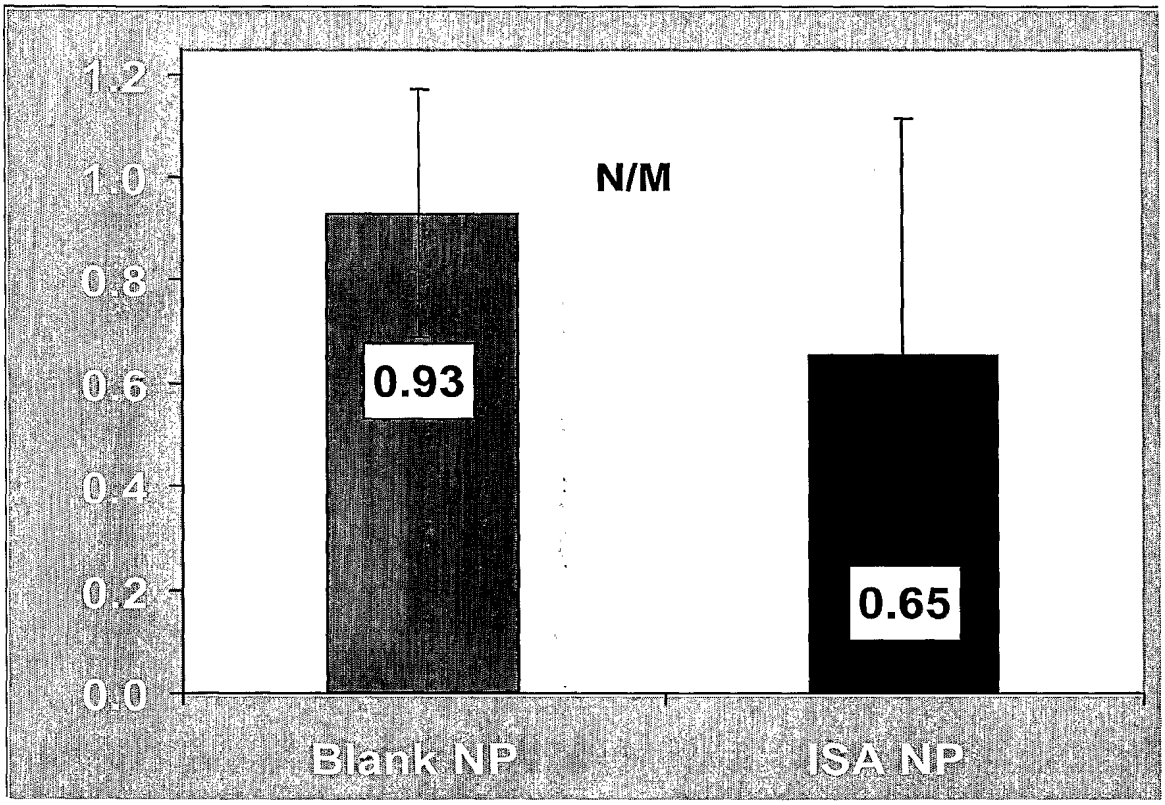


FIG. 8B

Formulation parameters	Alendronate NP (n=7)
Size (nm)	219±10
Alendronate entrapment (% of initial)	55.09±7.40
Alendronate in NP (mg/ml)	1.002±0.135
Alendronate in supernatant (mg/ml)	0.232±0.138
Initial amount of alendronate (mg)	20
PLGA amount (mg)	90
Volume of calcium chloride 0.246M (ml)	0.5

FIG. 9

Effect of Alendronate NP on RAW264 proliferation	
Concentration (µm)	**% inhibition (vs. control)**
1	3.60±1.3
5	13.53±4.24
10	25.73±12.33
50	98.14±0.04

FIG. 10

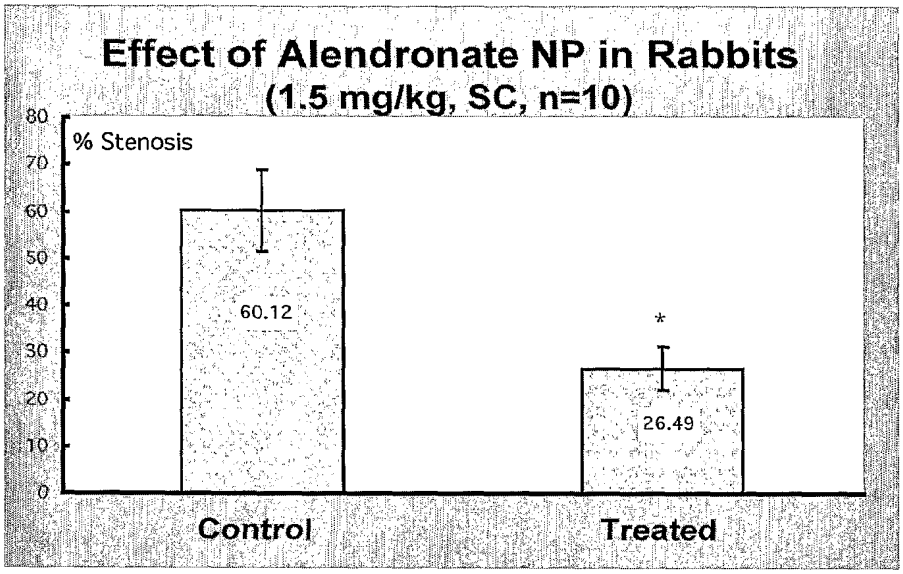

Effect of Alendronate NP in Rabbits
(1.5 mg/kg, SC, n=10)
% Stenosis
80
70
60
50
40
30
20
10
0
60.12
*
26.49
Control
Treated

FIG. 11

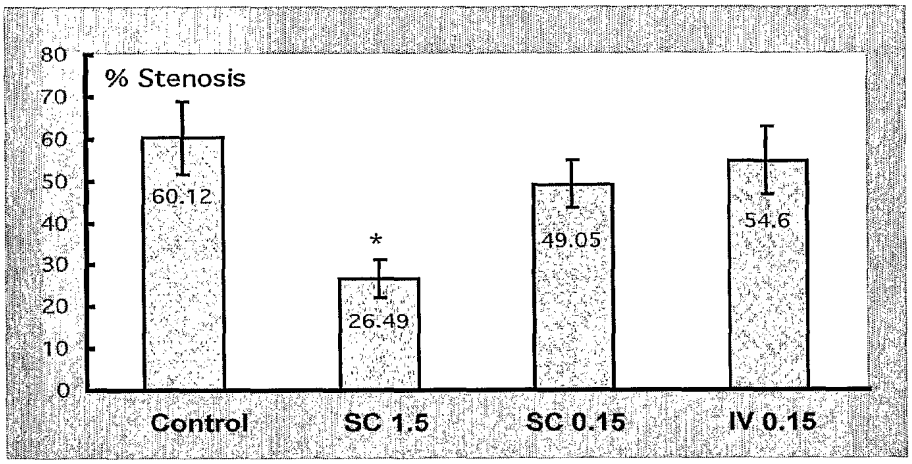


FIG. 12A

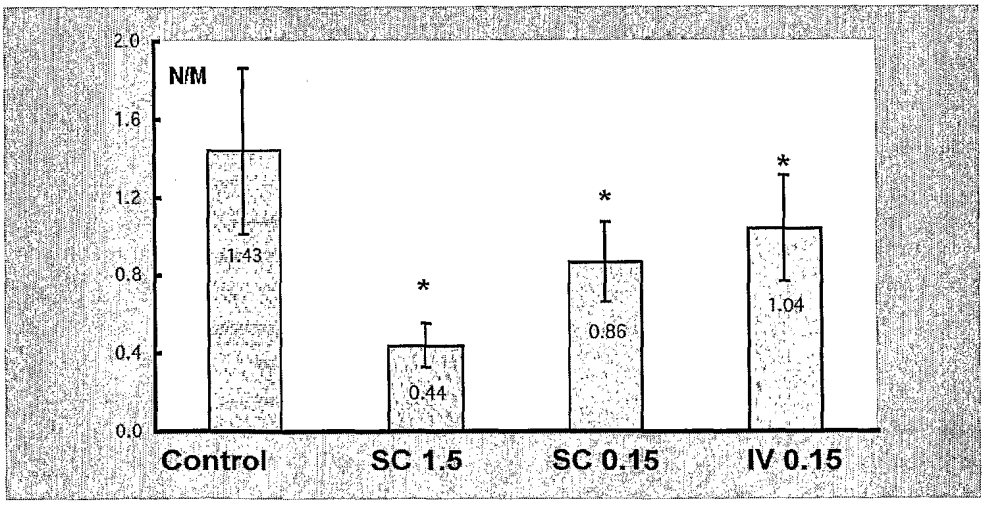


FIG. 12B

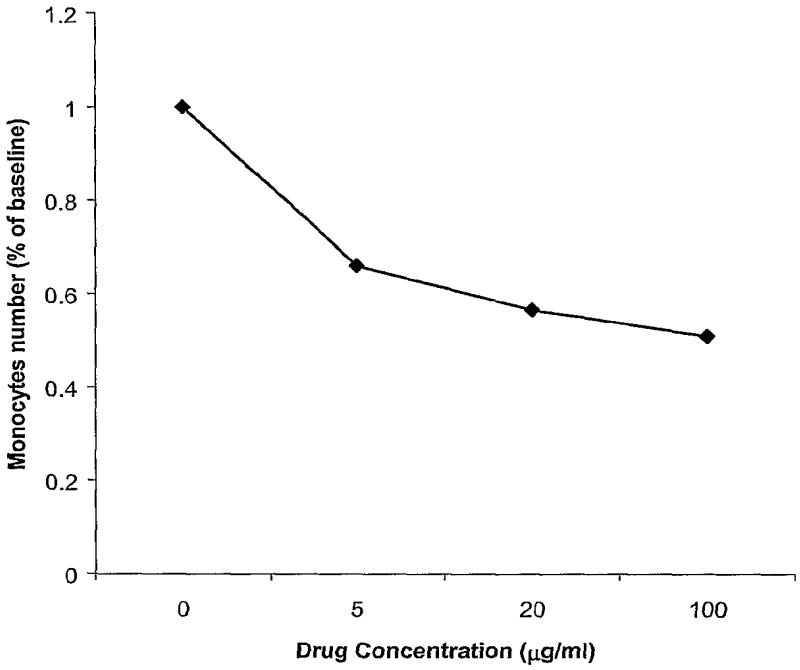

1.2
1
0.8
0.6
0.4
0.2
0
Monocytes number (% of baseline)
0
5
20
100
Drug Concentration (μg/ml)

FIG. 13

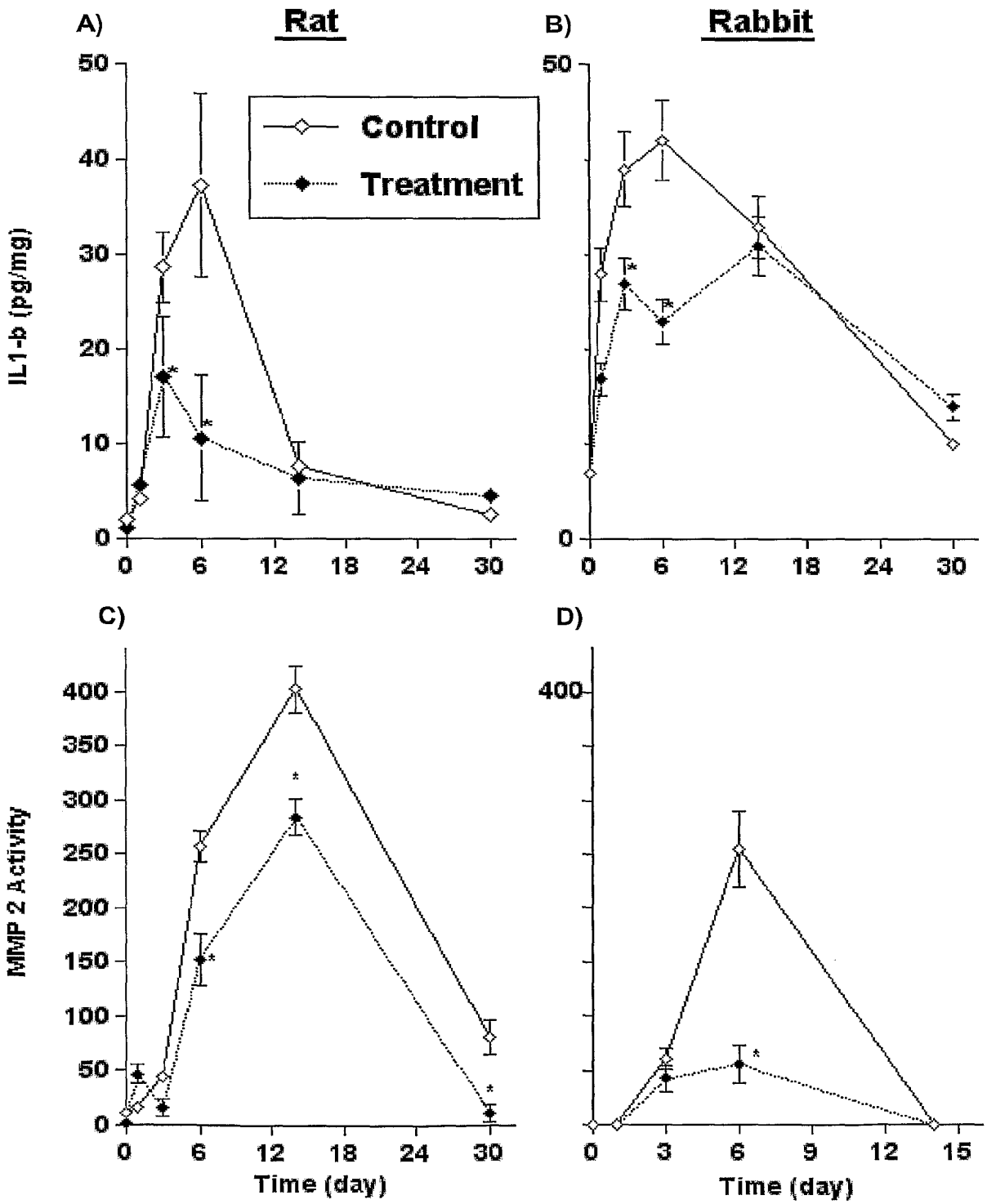

A)
Rat
B)
Rabbit
Control
Treatment
IL1-b (pg/mg)
C)
D)
MMP 2 Activity
Time (day)
Time (day)

FIG. 14

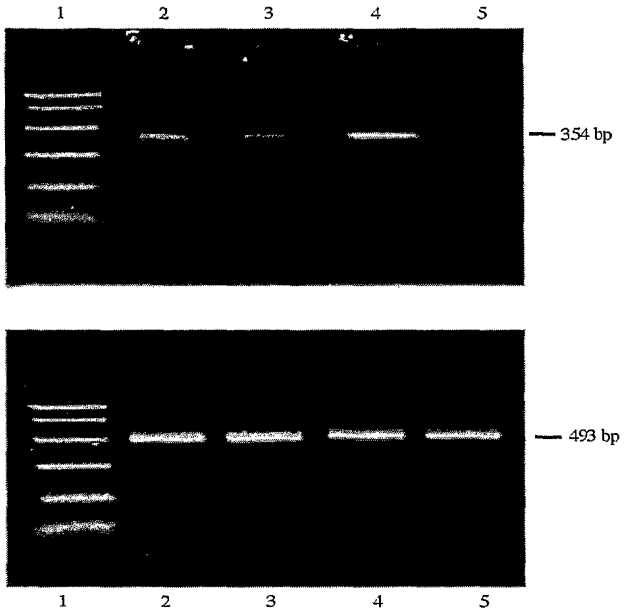


FIG. 15

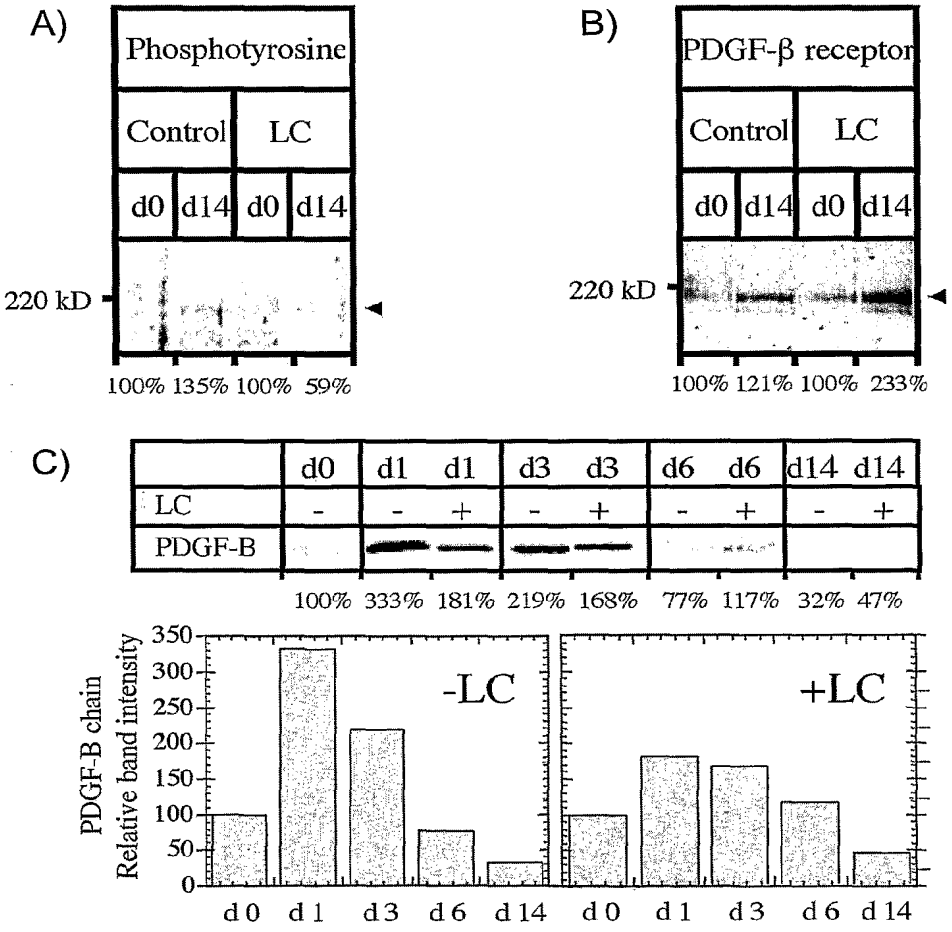

A)
Phosphotyrosine
Control
LC
d0
d14
d0
d14
220 kD
100% 135% 100% 59%
B)
PDGF-β receptor
Control
LC
d0
d14
d0
d14
220 kD
100% 121% 100% 233%
C)
d0
d1
d1
d3
d3
d6
d6
d14
d14
LC
-
-
+
-
+
-
+
-
+
PDGF-B
100% 333% 181% 219% 168% 77% 117% 32% 47%
PDGF-B chain
Relative band intensity
350
300
250
200
150
100
50
0
-LC
+LC
d 0
d 1
d 3
d 6
d 14
d 0
d 1
d 3
d 6
d 14

FIG. 16

SEQUENCE LISTING

<110> Yissum Research Development Company of the Hebrew University of Jerusalem

<120> Method of Inhibiting Restenosis

<130> 4313-4002PC

<140> TBA
<141> 2003-04-16

<150> US 10/126,248
<151> 2002-04-19

<150> US 10/160,207
<151> 2002-05-30

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<170> PatentIn version 3.2

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20

<210> 4
<211> 20
<212> DNA
<213> artificial

<220>
<223> b-actin antisense primer

<400> 4
ggatgtccac gtcgcacttc
20